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Development of Potent Thrombin Receptor Antagonist Peptides

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A peptide-based structure–activity study is reported leading to the discovery of novel potent thrombin receptor antagonists. Systematic substitution of nonproteogenic amino acids for the second and third residues of the human thrombin receptor "tethered ligand" sequence (SFLLR) led to a series of agonists with enhanced potency. The most potent pentapeptide agonist identified was Ser-*p*-fluoroPhe-*p*-guanidinoPhe-Leu-Arg-NH_2, **9** ($EC_{50} \sim 0.04\ \mu M$ for stimulation of human platelet aggregation, ~10-fold more potent than the natural pentapeptide). Systematic substitution of the NH_2-terminal Ser in **9** with neutral hydrophobic NH_2-acyl groups led to partial agonists and eventually antagonists with unprecedented potency (greater than 1000-fold increase over the previously reported antagonist 3-mercaptopropionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH_2). In the series of NH_2-acyl tetrapeptide antagonists, *N*-*trans*-cinnamoyl-*p*-fluoroPhe-*p*-guanidinoPhe-Leu-Arg-NH_2, **41** (BMS-197525), was identified as the tightest binding ($IC_{50} \sim 8$ nM) and most potent with an $IC_{50} \sim 0.20\ \mu M$ for inhibition of SFLLRNP-NH_2-stimulated platelet aggregation. Systematic single substitutions in **41** indicated that, in addition to the NH_2-terminal acyl group, the side chains at the second and third positions were also responsible for important and specific receptor interactions. The *p*-fluoroPhe and *p*-guanidinoPhe residues in the second and third positions of **41** were observed to be optimal in both the agonist and antagonist series. In the case of antagonists, however, an appropriately positioned positively charged group (i.e., protonated base) at the third residue was required. In contrast, such a substitution was not required for potent agonist activity. An even more potent antagonist resulted when **41** was extended at the C-terminus by a single Arg residue giving rise to analog **90** (BMS-200261) which had an $IC_{50} \sim 20$ nM for inhibition of SFLLRNP-NH_2-stimulated platelet aggregation. When the C-terminal Arg of **90** was replaced by an Orn-(N^5-propionyl) residue, the resulting antagonist **91** (BMS-200661) was suitable for use in radioligand binding assays (K_d = 10–30 nM). Antagonist activity observed for selected compounds was verified through secondary assays in that these analogs prevented SFLLRNP-NH_2-stimulated GTPase activity in platelet membranes and Ca^{2+} mobilization in cultured human smooth muscle cells and mouse fibroblasts. Furthermore, this inhibition occurred at concentrations that had no effect on thrombin catalytic activity indicating a specific activity attributable to receptor binding and not enzyme inhibition.

Introduction

Thrombin has been shown to have a variety of cellular actions that are mediated by proteolytic activation of a specific cell surface receptor known as the thrombin receptor (or "tethered ligand" receptor). The thrombin receptor is characterized by seven membrane-spanning domains[1] and is a member of the superfamily of G-protein-coupled receptors.[2] Activation of the receptor occurs by thrombin cleavage of an extracellular N-terminal domain thereby exposing a new NH_2-terminus which acts as a "tethered ligand" that intramolecularly binds to an appropriate site contained in the receptor structure.[1,3] By virtue of this structural rearrangement, the receptor becomes activated giving rise to various observable G-protein-coupled signal transduction pathways.[4]

The proteolytically activated thrombin receptor has been cloned[1,5–7] and shown to be present on numerous different cell types including human platelets,[1] endothelial cells,[6,8] fibroblasts,[5] vascular smooth muscle,[7,9] and cardiac myocytes.[10,11] A variety of responses are observable when such cells are treated with thrombin, and many of these actions can be mimicked by synthetic peptides corresponding to the N-terminal tethered ligand sequence. The identification of the thrombin receptor in relevant cell types, together with the realization that many of the pathophysiological actions of thrombin on these cells appear to be at least in part mediated by the thrombin receptor, suggests a role for this receptor in the pathological processes of thrombosis, inflammation, atherosclerosis, and fibroproliferative disorders.[1,5–11]

The development of thrombin receptor antagonists that may have value as therapeutic agents by specific inhibition of the cellular actions of thrombin (as opposed to its actions on clotting proteins) has been recognized,[4,12] but such an agent has not thus far been realized. Although limited peptide-based antagonists have been developed by analogy to the NH_2-terminal tethered ligand peptide of the thrombin receptor,[12–14] these compounds suffer serious limitations, specifically, lack of potency, partial agonist activity, and/or specificity.[4a]

An important objective of the work described in this report was to develop sufficiently potent, specific, and

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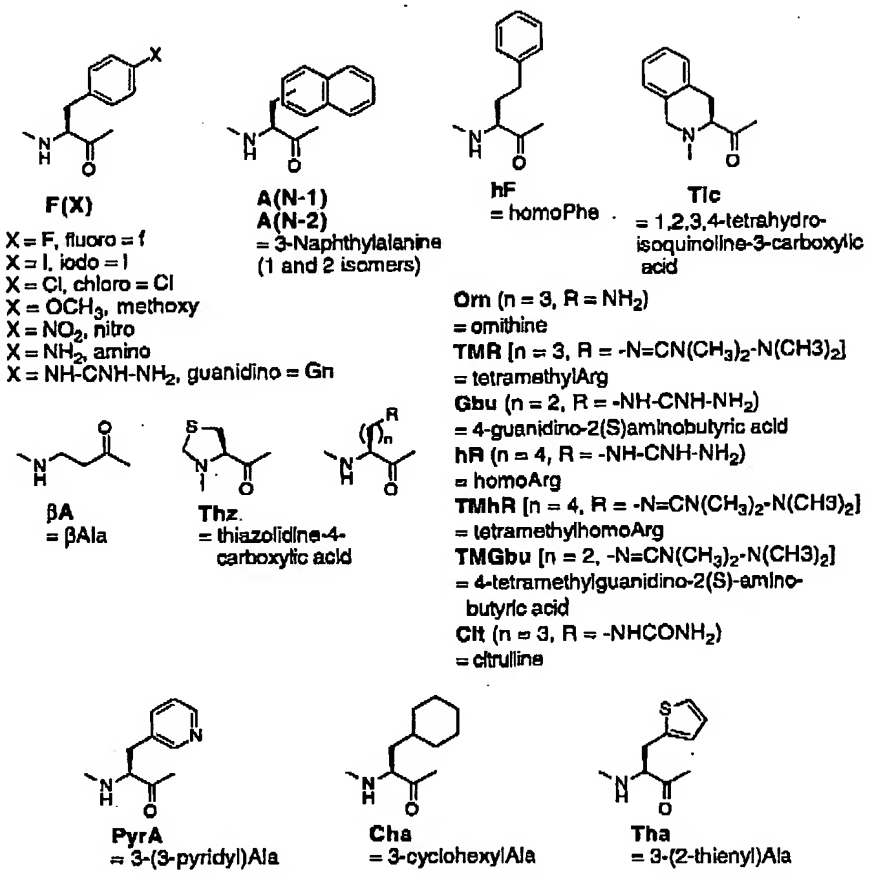

Figure 1. Structures and abbreviations used for nonproteogenic amino acid residues examined in the course of this study.

stable thrombin receptor antagonists that could be used for evaluation of their therapeutic potential.

In the abscence of precise structural information for the receptor, an "analog approach" was taken to antagonist development which involved cycles of systematic synthesis and testing. Previous studies of various thrombin receptor ligand peptides,[3,12,13,15-22] including a reported peptide-based structure–activity relationship (SAR) in which almost all of the natural amino acids were substituted, one at a time, for each residue of the natural biologically active "tethered ligand" sequence (SFLLR-NH_2, 2), provided a basic understanding of the peptide ligand side chain structural requirements and their relative tolerances with regard to their interactions with the human thrombin receptor.[21] Although very few of these single amino acid substitutions produced a significant increase in agonist potency, an important finding was that substitution of the nonproteogenic 3-(2-naphthyl)alanine residue for leucine at position 3 of the natural sequence produced by far the most potent agonist (~4-fold > 2) for human platelet aggregation found in that study.[21] A separate independent study indicated that substitution of *p*-fluorophenylalanine for phenylalanine at position 2 in SFLLRNP-NH_2 resulted in a 4–5-fold increase in activity as demonstrated by an assay measuring phosphoinositide turnover in human epithelial-like SH-EP cells.[20] The analog approach taken here was largely based upon additional substitution(s) with untested nonproteogenic amino acids.

This structure–activity excercise was expected to provide important information pertaining to (1) the elucidation of receptor binding mechanism(s), (2) the design and development of structural probes (e.g., photoaffinity labels), (3) the identification of peptides suitable for use to begin "computationally directed" screening of compound libraries for novel ligand types, (4) the development of novel antagonist-based binding assays (e.g., using an antagonist radioligand) for screening purposes, and (5) a basis for the design and development of novel nonpeptide antagonists. These objectives have at least been partially realized as a result of this study.

Results and Discussion

Agonist Optimization. Early on, the strategy employed here was to optimize agonist activity and then convert these to antagonists, if possible, by appropriate NH_2-terminal structural modification. Previous studies suggested that the use of nonproteogenic amino acids merited further exploration. Specifically, replacements with optimized unnatural residues, either singly or multiply in appropriate combination, might lead to further enhancements in binding and potency for known agonists. Furthermore analogs with nonproteogenic amino acid residues could have enhanced stability to proteases, an advantage if the peptides are to be studied in a biological setting. Figure 1 provides structures and

Table 1. Effect of Substitutions in the 2-Position of SFLLR-NH_2

peptide	mean EC_{50} (μM) (N)[a]
1. SF(I)LLR-NH_2	0.13 ± 0.05 (3)
2. SFLLR-NH_2 (human sequence)	0.40 ± 0.14 (3)
3. SF(OCH_3)LLR-NH_2	0.72 ± 0.15 (3)
4. SF(I)LLR-NH_2	9.27 ± 5.97 (3)
5. SF(NH_2)LLR-NH_2	18.00 ± 0.00 (3)
6. S-Tic-LLR-NH_2	110 ± 78.1 (3)
7. SF(Gn)LLR-NH_2	>300 (3)
8. S-hF-LLR-NH_2	>300 (3)

[a] Determined by platelet aggregation assay: EC_{50} = concentration required to stimulate 50% of maximum platelet aggregation. N = number of determinations. For amino acid abbreviations, see Figure 1.

Table 2. Effect of Substitutions at the 3-Position of Peptide 1

peptide	mean EC_{50} (μM) (N)[a]
9, SF(I)F(Gn)LR-NH_2	0.04 ± 0.02 (3)
10. SF(I)F(I)LR-NH_2	0.10 ± 0.03 (3)
11. SF(I)A(N-2)LR-NH_2	0.08 ± 0.04 (3)
12. SF(I)A(N-1)LR-NH_2	0.16 ± 0.06 (3)
13. SF(I)-Tic-LR-NH_2	0.47 ± 0.19 (6)

[a] See Table 1.

abbreviations for the various nonproteogenic residues examined in the course of this study.

Table 1 summarizes the results of platelet aggregation assays on a series of peptide agonists in which the Phe residue at position 2 of the human sequence was replaced by various aromatic side chain-containing analogs. These data show that the binding pocket for the second side chain is exquisitely sensitive to size, conformational constraint, and electronic distribution. Of all the substitutions made in this series, only the *p*-fluoroPhe residue provided an enhancement in potency over the wild type human sequence 2. The magnitude of the observed enhancement is in good agreement with previous reports for similar peptides.[20,22] It is clear from this and previously reported data[21] that the Phe binding site is very specific and that the *p*-fluoroPhe residue may represent a very nearly optimum substitution at position 2.

Table 2 summarizes the results of platelet aggregation assays on a series analogs with dual substitutions: the "optimized" *p*-fluoroPhe in position 2 and various residues at position 3. Substitution with both the *p*-fluoroPhe and 2-naphthylAla residues in peptide 11 produced an agonist with slightly enhanced potency (EC_{50} of 0.08 ± 0.04 μM) over that observed for either substitution alone (for reference, SFA(N-2)LR-NH_2 had an EC_{50} of 0.13 ± 0.07 μM).[21]

The *p*-guanidinoPhe replacement in the third position of peptide 9 was designed based on previous observations that a Phe substitution produced a peptide slightly greater in potency (~1.4-fold) than 2 and that an Arg substitution produced a somewhat greater (~1.7-fold) enhancement.[21] The *p*-guanidinoPhe residue was thus conceived as a hybrid combining potentially important structural aspects of the activity-enhancing residues: Phe, Arg, and 2-naphthylAla; this substitution in 9 produced the most potent peptide of this series with full agonist activity. As a result peptide 9 was utilized as a reasonable basis structure for further structural elaboration aimed at a crossover to antagonist activity, an approach that proved to be successful.

The results obtained for peptide 9 also suggested further investigation of minimum structural requirements for activity. The des-Arg tetrapeptide analog of 9 (SF(I)F(Gn)L-NH_2, 14, BMS-194021) was synthesized and found to be an agonist with an EC_{50} of 0.28 ± 0.08 μM (N = 7). This is the most potent tetrapeptide agonist thus far published and more potent than the natural pentapeptide 2. In contrast, the natural tetrapeptide SFLL-NH_2 was >400 times less potent than pentapeptide SFLLR-NH_2.[18] This shows that peptides with appropriate side chains as small as four residues can possess full activity and that five residues are not required as was previously thought.

Table 3. Effect of Preliminary N-Terminal Substitutions

peptide	mean EC_{50} (μM) (N)[a]
15. Ac-βAF(I)A(N-2)LR-NH_2	0.27 ± 0.03 (3)
16. Ac-βAFLLR-NH_2	1.80 ± 0.87 (3)
17. βAFLLR-NH_2	11.7 ± 4.51 (3)
18. Thz-FLLR-NH_2	46.0 ± 14.0 (3)
19. Ac-Thz-FLLR-NH_2	>300 (3)

[a] See Table 1.

It is also noteworthy that the conformational constraint imposed by a Tic residue in the third position of the peptide chain in analog 13 was relatively well tolerated, providing an analog nearly equal in potency to the natural sequence 2 but nonetheless substantially less potent than the other analogs of the series (Table 2).

Partial Agonist/Antagonist Discovery. Antagonists with modest potency had been reported which were based on the agonist structure where the Ser was replaced by a 3-mercaptopropionyl (Mpa) group[12,13] which suggested that antagonists might be achieved by substitution of Ser with neutral hydrophobic *N*-acyl groups. Initially derivatives of βAla and (*R*)-thiazolidine-4-carboxylic acid (Thz) were synthesized as near isosteric and conformationally constrained replacements for the Mpa group, respectively. The results are presented in Table 3. Very interestingly *N*-acetyl-βAla peptides **15** and **16**, although less potent than their Ser-containing counterparts, were found to be reasonably potent agonists. This result was somewhat surprising because, prior to this discovery, only peptides capable of providing a positively charged NH_2-terminus (i.e., a protonated amino group) were found to have appreciable agonist activity. Curiously, free amine peptide **17** was actually found to be less active in stimulation of platelet aggregation than its *N*-acetyl counterpart **16** (Table 3). This prompted additional biological evaluation including examination by GTPase assay.[21,23] Some peptides were capable of stimulating only a fraction of the total GTPase activity attainable by saturating levels of agonist peptide SFLLRNP-NH_2. For example, peptides **15**, **16**, and **18** were identified as partial agonists possessing 21%, 50–70%, and 33% intrinsic potency, respectively. This prompted further evaluation of NH_2-terminal substitutions in **9**. In the process, additional partial agonists and novel pure antagonists were identified. The antagonists were characterized by their ability to inhibit [^{3}H]SFFLRR-NH_2 binding to platelet membranes,[24] inability to stimulate platelet aggregation at concentrations >300 μM, and dose dependent inhibition of peptide agonist (or thrombin)-induced platelet aggregation, as well as their inability to stimulate the GTPase even at very high concentration. Tables 4 and 5 summarize these results.

Table 4. Effect of N-Terminal Variations in Early Generation Antagonist Peptides: X-F(f)F(Gn)LR-NH_2

peptide	X	IC_{50} (μM)[a] (N)	IC_{50} (μM)[b] (N)
20	(2-thiophene)acetyl	0.26 (2)	32 ± 23 (4)
21	N-acetyl-2-aminobenzoyl	1.00 ± 0.31 (3)	ND
22	2-oxo(2-thiophene)acetyl	0.22 ± 0.08 (3)	47 ± 32 (4)
23	(3-thiophene)acetyl	0.31 ± 0.18 (3)	74 ± 38 (4)
24	phenylacetyl	0.68 ± 0.42 (3)	94 ± 37 (3)
25	(2-thiophene)sulfonyl	8.95 (2)	193 ± 23 (3)
26	(3-fluorophenyl)acetyl	0.16 ± 0.05 (3)	40 ± 19 (4)
27	(4-fluorophenyl)acetyl	0.35 ± 0.16 (3)	68 ± 17 (3)
28	3-pyridylacetyl	0.95 ± 0.34 (3)	>160 (4)
29	(2-fluorophenyl)acetyl	0.56 ± 0.14 (3)	35 ± 11 (4)
30	(3-indole)acetyl	0.50 ± 0.10 (3)	54 ± 8 (4)
31	cyclopentylacetyl	0.37 ± 0.17 (3)	85 ± 26 (4)
32	2-oxo(3-indole)acetyl	0.13 ± 0.08 (3)	9.9 ± 6.5 (3)
33	3-indoloyl	0.019 ± 0.011 (3)	2.0 ± 0.7 (3)
34	(3-chlorophenyl)acetyl	0.11 (2)	2.9 ± 1.1 (3)

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] IC_{50} = concentration required for 50% inhibition of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation.

Table 5. Effect of N-Terminal Structure (X) on Peptides (X-F(f)F(Gn)LR-NH_2) Identified as Partial Agonists

peptide	X	IC_{50} (μM)[a] (N)	EC_{50} (μM)[b] (N)
35	N-acetyl-4-aminobutyryl	0.27 ± 0.16 (3)	8.7 (3)
36	2-thiopheneoyl	0.025 ± 0.014 (3)	0.10 ± 0.07 (4)
37	3-thiopheneoyl	0.024 ± 0.008 (3)	PA[c]
38	3-furanoyl	0.029 ± 0.004 (3)	PA[c]
39	2-indoloyl	0.024 ± 0.003 (3)	1.8 (2)
40	4-chlorobenzoyl	0.026 (2)	3.7 (2)

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] EC_{50} = concentration required for stimulation of 50% maximum platelet aggregation. [c] PA = partial agonists for which maximum platelet aggregation was not experimentally achieved.

In the course of generating and evaluating the data (Tables 4 and 5), simple generalizations became possible. Whenever a relatively small aryl ring system was directly attached to the carbonyl group of the NH_2-terminal amide, partial agonists resulted. Inclusion of a spacer group such as a methylene or additional carbonyl group between the aryl ring and the amide carbonyl resulted in antagonists. From this data it was hypothesized that antagonists that provided a structure capable of extending greater distances from the NH_2-terminal amide, possibly with some conformational constraint, could result in enhanced binding by virtue of additional favorable interactions with the receptor that are unavailable to shorter NH_2-termini. To test this possibility an NH_2-terminal *trans*-cinnamoyl group, containing a conformationally restrictive and chain-extending olefinic linker between an aryl ring and the N-terminal amide group, was incorporated into an analog in this series (peptide **41**). Upon its biological evaluation, peptide **41** (Table 6) was found to be the tightest binding and most potent NH_2-acyl tetrapeptide antagonist thus far discovered. The antagonist activity of compound **41** was confirmed through additional activity assays (for more details, see Antagonist Validation section). Table 6 summarizes data obtained for **41** and related NH_2-acyl tetrapeptide analogs, many of which were also found to be reasonably potent antagonists.

Antagonist Development. Despite the relatively large number of NH_2-acyl group replacements for the *trans*-cinnamoyl moiety in **41** (Tables 4–6), only conservative modifications of the NH_2-terminal cinnamoyl group of **41** were well tolerated. NH_2-Terminal structures giving rise to antagonists with binding assay IC_{50} values <50 nM are given in Figure 2. These results indicate a high degree of structural specificity for the receptor binding site interacting with the NH_2-termini of these analogs and an important functional role for this interaction dictating the type of activity observed for a particular analog (agonist vs partial agonist vs antagonist). The data also suggest that the phenyl ring in the cinnamoyl group in **41** binds with a preferred conformation (twisted out of the plane of the adjacent olefin), as more rigid but reasonably conservative analogs **53** and **54** demonstrate dramatic reductions in binding and potency.

Another specific receptor interaction important for both binding and antagonist function was recognized by substituting the *p*-guanidinoPhe residue of **41** with various aromatic, neutral, or basic residues (Tables 7–9). Here again, the lead analog **41** remained the tightest binding and most potent antagonist. These data indicate a requirement for a positively charged group (protonated amine or guanidine) in order to obtain tight binding antagonist activity. Replacement of the guanidino group of **41** with hydrogen (analog **69**) produced a very significant reduction in binding and, remarkably, a crossover to some agonist activity. Appropriate positioning of the charge with respect to the rest of peptide as well as the size of the charged group are also important factors influencing binding and function. For example, Lys, homoArg, and *N,N'*-tetramethylhomoArg relacements (analogs **79–81**) gave rise to partial agonist activity, while replacement with Arg (**76**) was better tolerated resulting in an antagonist with slightly reduced affinity and potency. These data, taken together with the SAR obtained for NH_2-terminal variants, suggest that the arylguanidine of **41** plays an important role in orienting or positioning the the NH_2-terminal cinnamoyl group for an optimum antagonist generating interaction with the receptor.

Effect of Peptide Length/Antagonist Radioligand Development. The effects of C-terminal deletions and additions or replacements in anlogs of lead peptide **41** were also examined (Tables 10 and 11). Very interestingly, binding at the sub-micromolar level was retained in a molecule as small as the NH_2-acyl dipeptide analog **83**, and the potency of this dipeptide was comparable to that of a number of NH_2-acyl tetrapeptide antagonists (Table 4) having less optimal NH_2-termini. Such results suggest that it may be possible to prepare relatively small molecule peptidomimetic thrombin receptor antagonists with useful potency. When the CO_2H-terminal Arg was deleted from **41** to give analog **84**, significant reduction in both binding and potency are observed but reasonable antagonist activity is retained. The contribution of the CO_2H-terminal Arg of **41** to binding and potency is nonetheless substantial.

SAR reported for peptide agonists[3,15–18,22] suggested that the binding and potency of **41** may be enhanced by the inclusion of an additional CO_2H-terminal residue; furthermore, if an Orn residue could be tolerated at this position, acylation of its side chain amine with a radiolabled acylating agent might provide a useful radioligand for development of antagonist-based binding assays. The results obtained for anlogs with CO_2H-terminal replacements or extensions are given in Tables

Table 6. Effect of N-Terminal Structure (X) on *N*-Acyl Tetrapeptide Series: X-F(f)F(Gn)LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	IC_{50} (μM)[b] (*N*)
41	*trans*-cinnamoyl	0.0078 ± 0.0033 (3)	0.20 ± 0.07 (4)
42	3-phenyl-2-propynoyl	0.023 ± 0.014 (3)	0.28 ± 0.20 (6)
43	*p*-fluoro-*trans*-cinnamoyl	0.020 ± 0.003 (3)	0.09 ± 0.04 (3)
44	*p*-chloro-*trans*-cinnamoyl	0.099 ± 0.006 (3)	0.24 ± 0.06 (6)
45	*p*-methyl-*trans*-cinnamoyl	0.034 ± 0.008 (3)	0.95 ± 0.83 (5)
46	*p*-methoxy-*trans*-cinnamoyl	0.040 ± 0.023 (3)	0.39 ± 0.13 (3)
47	4-biphenyloyl	0.046 ± 0.014 (3)	0.43 ± 0.25 (5)[c]
48	*m*-chloro-*trans*-cinnamoyl	0.041 ± 0.020 (3)	0.08 ± 0.04 (4)[d]
49	3-phenylpropionyl	0.042 ± 0.007 (3)	1.19 ± 0.56 (5)
50	phenoxyacetyl	0.021 ± 0.003 (3)	0.26 ± 0.08 (4)
51	1-naphthylacetyl	1.63 ± 0.67 (3)	3.87 ± 1.94 (3)
52	3-(2-thiophene)-*trans*-acryloyl	0.023 ± 0.003 (3)	0.88 ± 0.44 (4)
53	(±)-*trans*-3-phenylcyclopropanoyl	2.32 ± 0.93 (3)	3.00 ± 1.31 (3)
54	3-coumarinoyl	4.33 ± 0.15 (3)	21.7 ± 14.5 (3)
55	4-phenylbutyryl	1.26 ± 1.00 (3)	3.93 ± 1.68 (3)
56	*p*-amino-*trans*-cinnamoyl	0.061 ± 0.013 (3)	1.63 ± 0.25 (3)
57	2-naphthylacetyl	0.295 ± 0.057 (3)	4.67 ± 1.92 (3)[e]
58	*p*-hydroxy-*trans*-cinnamoyl	0.062 ± 0.015 (3)	2.73 ± 0.58 (3)
59	(thiophenoxy)acetyl	0.019 ± 0.007 (3)	0.69 ± 0.16 (3)
60	*trans*-2-*trans*-4-hexadienoyl	0.024 ± 0.009 (3)	PA[f]
61	*trans*-2-octenoyl	0.035 ± 0.013 (3)	7.60 ± 9.02 (3)
62	α-fluorocinnamoyl	0.023 ± 0.008 (3)	0.53 ± 0.27 (3)
63	α-methylcinnamoyl	0.043 ± 0.001 (3)	8.33 ± 6.81 (3)
64	α-phenylcinnamoyl	1.53 ± 0.15 (3)	66.7 ± 11.6 (3)

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] IC_{50} = concentration required for 50% inhibition of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation. [c-e] These peptides displayed well-resolved mixed pharmacology with agonist (platelet aggregation) activity EC_{50}'s of [c] 18 μM ($N = 2$), [d] 120 μM ($N = 2$), and [e] 90 μM ($N = 1$). [f] PA = partial agonist for which maximum platelet aggregation was not experimentally achieved.

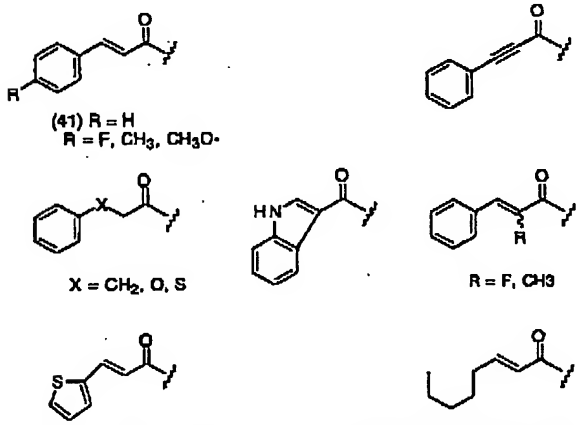

Figure 2. Best tolerated (IC_{50} < 50 nM, binding) N-terminal structures in antagonist analogs of **41**.

Table 7. Effects of Hydrophobic Aromatic Replacements (X) for the *p*-GuanidinoPhe Residue in Analogs of **41**: *trans*-Cinnamoyl-F(f)-X-LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	IC_{50} (μM)[b] (*N*)
65	homoPhe	0.63 ± 0.026 (3)	>300 (2)
66	*p*-nitroPhe	7.55 ± 10.4 (4)	>300 (2)
67	*p*-chloroPhe	4.71 ± 1.0 (3)	>300 (2)
68	*p*-methoxyPhe	1.18 ± 0.13 (3)	>300 (2)
69	Phe	1.12 ± 0.33 (4)	17 (2)[c]
70	β-(2-naphthyl)Ala	0.246 ± 0.018 (3)	5 (1)[c]

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] IC_{50} = concentration required for 50% inhibition of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation. [c] These peptides displayed agonist activity with the specified EC_{50} values.

11 and 12. Addition of a basic residue (Orn or Arg in analogs **88** and **90**, respectively) resulted in analogs with marginally improved binding but a more significant positive impact on antagonist potency (about a 5–10-fold enhancement over **41**) was observed. When the Orn residue of **88** was acetylated (**89**) or propionylated (**91**), a slight reduction in binding (<3-fold) was observed, but these analogs were still more potent at inhibiting platelet aggregation than **41**. Given these results, peptide **91** was targeted for radioligand synthesis. After propionylation of **88** in solution with *N*-succinimidyl-[2,3-^{3}H]propionate (Amersham), HPLC-purified tritiated **91** was obtained with a specific activity of 80 Ci/mmol and a radiochemical purity of >98% (P. Egli, unpublished results). Using this tritiated **91**, an antagonist-

Table 8. Effects of Hydrophobic Neutral or Weakly Basic Aromatic Replacements (X) for the *p*-GuanidinoPhe Residue in Analogs of **41**: *trans*-Cinnamoyl-F(f)-X-LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	EC_{50} (μM)[b] (*N*)
71	citrulline	1.04 ± 0.06 (4)	67 (2)
72	His	0.47 ± 0.11 (3)	35 ± 22 (4)[c]
73	β-(3-pyridyl)Ala	0.34 ± 0.02 (3)	116 ± 100 (4)[c]
74	*p*-aminoPhe	0.37 ± 0.03 (3)	60

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] EC_{50} = concentration required for stimulation of 50% maximum platelet aggregation. [c] Concentration required for 50% inhibition (i.e., IC_{50}) of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation.

Table 9. Effects of Aliphatic Basic (i.e., Positively Charged) Residue Replacements (X) for the *p*-GuanidinoPhe Residue in Analogs of **41**: *trans*-Cinnamoyl-F(f)-X-LR-NH_2

peptide	X	IC_{50} (μM)[a] (*N*)	IC_{50} (μM)[b] (*N*)
75	Orn	0.17 ± 0.08 (3)	8.70 ± 3.63 (4)
76	Arg	0.028 ± 0.016 (3)	1.13 ± 0.67 (3)
77	TMR	0.32 ± 0.13 (3)	16 (2)
78	Gbu	0.028 ± 0.007 (3)	8.0 (1)
79	Lys	0.039 ± 0.002 (3)	18 (1)[c]
80	hR	0.012 ± 0.002 (3)	0.5 (1)[c]
81	TMhR	0.13 ± 0.03 (3)	90 (1)[c]
82	TMGbu	0.34 ± 0.18 (3)	140 (1)

[a] IC_{50} = concentration required to inhibit 50% of tritiated agonist (SFFLRR-NH_2, at 25 nM) binding. [b] IC_{50} = concentration required for 50% inhibition of agonist (SFLLRNP-NH_2, at 3 μM)-induced platelet aggregation. [c] These peptides displayed agonist (or partial agonist) activity with the specified EC_{50} values.

Table 10. Effects of C-Terminal Truncation in Analogs of 41: *trans*-Cinnamoyl-F(f)-F(Gn)-X

peptide	X	IC_{50} (μM)[a] (N)	IC_{50} (μM)[b] (N)
83	NH_2	0.78 ± 0.20 (3)	64 (2)
84	Leu-NH_2	0.11 ± 0.09 (3)	4.05 ± 2.66 (4)
85	Orn-NH_2	0.87 ± 0.13 (3)	141 (2)

[a,b] See Table 9.

Table 11. Effects of C-Terminal Replacements and Additions to Analogs of 41: *trans*-Cinnamoyl-F(f)-F(Gn)L-X

peptide	X	IC_{50} (μM)[a] (N)	IC_{50} (μM)[b] (N)
86	Orn-NH_2	0.017 ± 0.003 (3)	0.19 ± 0.09 (3)
87	Orn(acetyl)-NH_2	0.044 ± 0.002 (3)	0.25 ± 0.22 (3)
88	Arg-Orn-NH_2	0.0066 ± 0.002 (3)	0.047 ± 0.018 (4)
89	Arg-Orn(acetyl)-NH_2	0.0166 ± 0.006 (3)	0.040 ± 0.020 (4)
90	Arg-Arg-NH_2	0.0075 ± 0.001 (3)	0.021 ± 0.004 (3)
91	Arg-Orn(pro)[c]-NH_2	0.0219 ± 0.004 (3)	0.085 ± 0.025 (4)

[a,b] See Table 9. [c] pro = propionyl.

based binding assay was developed (S. M. Seiler, unpublished results) showing competitive binding with various agonists and antagonists, as well as distinctly different kinetics of binding compared to the normal agonist radioligand. Those studies will be detailed separately.

Results of replacing the NH_2-terminal *trans*-cinnamoyl group in the most potent analog **90** with some of the best tolerated replacements identified previously in the NH_2-acyl tetrapeptide series (Table 6, Figure 2) are given in Table 12. In this case, the NH_2-terminal 3-phenyl-2-propynoyl group was found to provide the tightest binding (IC_{50} ~ 4 nM) antagonist **94** (BMS-201516) thus far discovered, but it was comparable in platelet aggregation inhibition potency to its *trans*-cinnamoyl counterpart **90**. Thus, in addition to a positively charged residue (e.g., *p*-guanidinoPhe) at position 3 possibly having some role in positioning the critical NH_2-terminus at the receptor binding site, such NH_2-terminal positioning may also be influenced by interactions sequentially more remote (i.e., at the more CO_2H-terminal side chains of positions 5 and 6). Overall the results reported here for CO_2H-terminally extended antagonists are consistent with and parallel those observed for peptide agonists for which optimal potency was observed at the length of hexapeptides.[22]

Effect of 2-Position Substitutions in Antagonists. Because the *p*-fluorophenyl side chain represented an optimal substitution at the 2-position in the peptide agonists, this specific and important interaction was further probed in the tight binding NH_2-acyl tetrapeptide antagonist series by various systematic substitutions. The results are presented in Table 13. As observed for agonist peptides, the *p*-fluorophenyl side chain provides an optimal and apparently specific and important interaction. Conservative substitution with a phenyl side chain in analog **97** was tolerated but resulted in significant losses in binding (~3-fold) and potency (>8-fold). Furthermore a single replacement of the *p*-fluorophenyl side chain of **41** by a hydrogen (Gly analog **96**) produced a peptide that was almost devoid of activity. Clearly a marked preference for a hydrophobic aromatic residue has been demonstrated at this position.

Antagonist Validation. Because of the unprecedented binding and antagonist activity observed for peptide **41**, additional biological characterization was carried out. Peptide **41** blocked platelet aggregation stimulated by 3 μM SFLLRNP-NH_2 with an IC_{50} of 0.2 ± 0.07 μM ($N = 4$) and caused concentration dependent rightward shifts in the concentration of agonist required for platelet aggregation (Figure 3), indicating that the compound is a competitive antagonist for SFLLRNP-NH_2 activation of platelets. The inhibition of platelet aggregation gave a Schild slope of 1.02 and a pA_2 of 7.26 ($N = 2$). This inhibition was specific to the thrombin receptor in that platelet aggregation stimulated by ADP and U-46619, a thromboxane receptor agonist, was unaffected. Peptide **41** also inhibited thrombin-induced platelet aggregation, but its potency against SFLLRNP-NH_2 was greater than against thrombin. The IC_{50} for inhibition of thrombin-induced platelet aggregation also depended on the thrombin concentration and the individual platelet donor. This result is consistent with enzymatic cleavage of the receptor generating a tethered ligand having a very high effective local concentration, thereby reducing the likelihood of inhibition by a competitive antagonist at less than saturating concentrations.

Antagonist **41** also inhibited SFLLRNP-NH_2 and thrombin-stimulated membrane GTPase activity in platelet and CHRF-288 cell membranes. The inhibition of SFLLRNP-NH_2-stimulated platelet membrane GTPase is shown in Figure 4. In human aortic smooth muscle cells (HASMs), most of the intracellular Ca^{2+} mobilization observed with SFLLRNP-NH_2 was prevented by **41** (Figure 5). As for the platelet aggregation studies, a higher concentration of **41** was required to inhibit HASM activation by thrombin compared to SFLLRNP-NH_2. Analog **41** also inhibited SFLLRNP-NH_2-stimulated Ca^{2+} mobilization in mouse Swiss 3T3 cells (data not shown). These and other studies indicate that the antagonists reported here inhibit the thrombin receptor in at least several different species and tissues.

Furthermore **41** was found not to inhibit thrombin proteolytic activity[25] (K_i = 52 μM for inhibition of thrombin by **41**) at levels where antagonist responses were observed showing that its biological activity was thrombin receptor specific.

Summary

Beginning with existing SAR obtained for thrombin receptor agonist peptides and preparing novel analogs by systematically substituting residues with nonproteogenic amino acids, first singly and then in combination, it was possible to derive the most potent agonist pentapeptide, SF(f)F(Gn)LR-NH_2 (**9**), reported to date. Single substitution of the NH_2-terminal Ser in agonist **9** with a wide variety of neutral hydrophobic NH_2-acyl groups led to the identification and discovery of novel partial agonists and antagonists. The most potent NH_2-acyl tetrapeptide antagonist derived from **9** was *trans*-cinnamoyl-F(f)F(Gn)LR-NH_2 (**41**) which exhibited specific binding to the human thrombin receptor at unprecedented single-digit nanomolar levels (> 1000-fold enhancement over reported peptide antagonists) and inhibited agonist-induced platelet aggregation with an IC_{50} potency of ~0.2 μM. Data obtained for NH_2-terminal acyl group substitution analogs of **41** suggest a specific and important interaction for the *trans*-cinnamoyl moiety of **41**. Further SAR by systematic substitutions of the *p*-fluoroPhe and *p*-guanidinoPhe residues of **41** did not lead to improved binding or antagonist potency indicating important and specific

Table 12. Effects of Single (X) or Dual (X, Z) Replacement in Analogs of 90: X-F(l)-F(Gn)-LR-Z-NH_2

peptide	X	Z	IC_{50} (μM)[a] (N)	IC_{50} (μM)[b] (N)
92	*trans*-4-fluorocinnamoyl	Arg	0.019 ± 0.007 (3)	0.05 ± 0.01 (3)
93	3-phenylpropionyl	Arg	0.009 ± 0.001 (3)	0.25 ± 0.12 (3)
94	3-phenyl-2-propynoyl	Arg	0.004 ± 0.001 (3)	0.040 ± 0.019 (3)
95	*trans*-4-fluorocinnamoyl	Orn(acetyl)	0.025 ± 0.005 (3)	0.060 ± 0.010 (3)

[a,b] See Table 9.

Table 13. Effects of Substitutions (X) for *p*-FluoroPhe in Analogs of 41: *trans*-Cinnamoyl-X-F(Gn)LR-NH_2

peptide	X	IC_{50} (μM)[a] (N)	IC_{50} (μM)[b] (N)
96	Gly	>30	>100 (2)
97	Phe	0.024 ± 0.007 (4)	1.73 ± 0.46 (3)
98	Cha[c]	0.093 ± 0.032 (4)	8.73 ± 4.95 (3)
99	Tha[c]	0.042 ± 0.011 (4)	3.31 ± 0.57 (3)
100	PyrA[c]	3.44 ± 1.14 (4)	>55 (3)

[a,b] See Table 9. [c] See Figure 1 for structures and abbreviations.

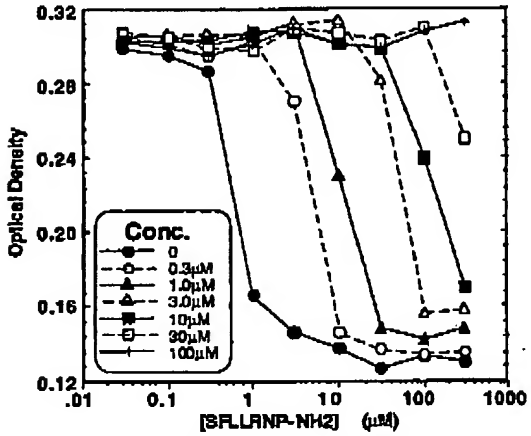

Figure 3. Inhibition of SFLLRNP-NH_2-stimulated human platelet aggregation by peptide 41. Platelet aggregation was measured using gel-filtered human platelets in a microtiter turbidity assay as previously described.[21,25] The platelets were preincubated with the indicated concentrations of 41 prior to stimulation by SFLLRNP-NH_2. This experiment is an example of several yielding similar results.

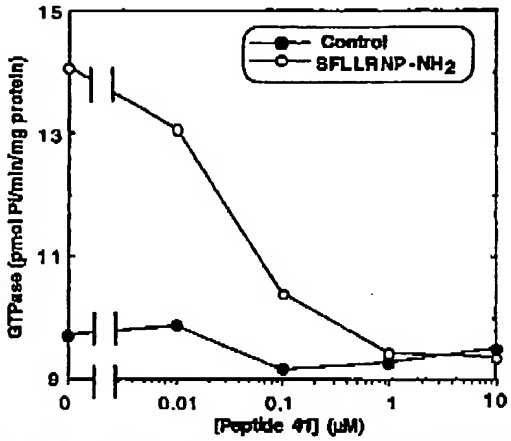

Figure 4. Inhibition of SFLLRNP-NH_2-stimulated platelet membrane GTPase activity by peptide 41. Platelet membrane GTPase was measured with (○) and without (●) 20 μM SFLLRNP-NH_2. Thrombin receptor-stimulated GTPase was determined as described previously.[23] This experiment is an example of three yielding similar results.

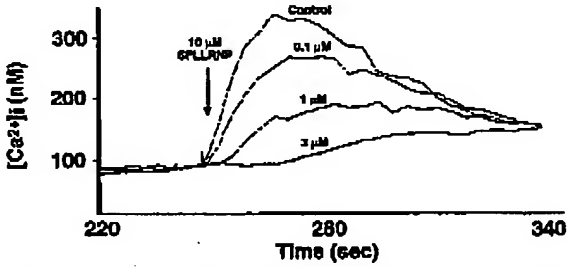

Figure 5. Inhibition of SFLLRNP-NH_2-stimulated Ca^{2+} mobilization in cultured human aortic smooth muscle (HASM) cells. The cells were loaded with the fura-2 Ca^{2+} fluorescent indicator by incubation with fura-2 AM. The loaded cells were washed and preincubated with the indicated concentrations of peptide 41. Ca^{2+} mobilization was induced by addition of 10 μM SFLLRNP-NH_2 (arrow). Fluorescence (excitation at 340 and 380 nm with emission at 505 nm) was measured.

interactions with the receptor at those peptide sites in addition to those identified at the NH_2-terminus. Also noteworthy is an apparent requirement for an appropriately positioned positively charged group (i.e., protonatable base) to mimic the guanidinophenyl side chain of 41 if tight binding and potent antagonism are to be realized. In general the antagonist SAR thus obtained parallels that obtained for agonists. These observations, combined with the ability of peptide agonists and antagonists to compete for receptor binding, suggest at least partially common modes of binding for both types with distinct and functional differences residing mainly at the NH_2-termini that dictate what type of activity (GTPase coupling or uncoupling, i.e., agonist or antagonist) is observed for a particular structure. Furthermore it appears that the critical NH_2-terminal interaction is at least in part influenced by interactions downstream with more CO_2H-terminal side chains. Thus, extension of 41 by inclusion of an additional Arg residue at the CO_2H-terminus gave the most potent antagonist 90 with similar receptor binding affinity to 41 but with a significant increase (~10-fold, IC_{50} ~ 20 nM) in potency. When the NH_2-terminal *trans*-cinnamoyl group of 90 was replaced with a 3-phenyl-2-propynoyl group, the tightest binding antagonist 94 (IC_{50} ~ 4 nM) resulted, which had potency comparable to 90. Given the specificity, unprecedented binding affinity, and potency for the thrombin receptor antagonists disclosed here, it is likely that these, or specifically designed derivatives of these, will serve as valuable tools for elucidation of thrombin receptor structure, function, and pharmacology and serve as a starting point for new drug discovery. Indeed, a practically accessible tritiated antagonist (91) has been shown to be a useful radioligand suitable for use in binding assays.

Experimental Section

Materials. Boc-Leu-OH, Boc-Arg(Tos)-OH, and *p*-methylbenzhydrylamino-polystyrene resins (1% DVB cross-linked,

0.56 and 0.36 mequiv/g) were purchased from Peninsula Laboratories (Belmont, CA). All nonproteogenic Boc-amino acids were from Bachem Bioscience (Philadelphia, PA) except Boc-Phe(*p*-*N,N'*-bis-Cbz-guanidino)-OH which was prepared by reaction of Boc-Phe(*p*-NH_2)-OH with *N,N'*-bis-Cbz-1-guamylpyrazole[26] as described previously.[27] Trifluoroacetic acid was from Eastman Kodak (Rochester, NY), diisopropylethylamine was from Fluka (Buchs, Switzerland), and BOP and HBTU reagents were from Midwest Biotech (Fishers, IN). Coupling reagent solution, 1 M DCC in NMP, was from Applied Biosystems (Foster City, CA). Carboxylic acids used for N-terminal acylations, 2-thiophenesulfonyl chloride, and anhydrous anisole were from Aldrich (Milwaukee, WI). Synthesis solvents (CH_2Cl_2, DMF, diethyl ether) were obtained from Fisher Scientific (Pittsburgh, PA). Hydrogen fluoride was from Matheson (East Rutherford, NJ). All commercial chemicals used were of the highest quality available (AR grade or better).

Peptide Synthesis. All peptides were prepared manually using standard solid phase synthesis techniques and the Boc/benzyl protection strategy. Syntheses were performed starting from 0.08–0.10 mmol of *p*-methylbenzhydrylamino-polystyrene resin. Boc group removals were carried out by treatment with TFA/CH_2Cl_2 (1:1) for 15 min. Neutralization was performed by two brief washes with 5% DIEA in CH_2Cl_2. Couplings were performed using 4 equiv of Boc-amino acid (or 3 equiv for nonproteogenic Boc-amino acids) with equivalent amounts of BOP reagent[28] and DIEA in minimum DMF and were monitored for completion using the Kaiser ninhydrin test.[29] NH_2-terminal acylations by carboxylic acids in general were conducted similarly (i.e., BOP/DIEA) except that 10 equiv were used. In some cases, where BOP-derived intermediates precipitated during preactivation, carboxylic acids were coupled using 1 M DCC in NMP. In the case of the NH_2-terminal phenylpropioloyl group (analogs 42, 94), N-acylation was conducted using the *N*-hydroxysuccinimide ester of phenylpropiolic acid (prepared by reaction of phenylpropiolic acid with DCC and *N*-hydroxysuccinimide in THF at 4 °C, 2 h, then at room temperature, 2 h) in CH_2Cl_2/NMP. In some cases, arginine residues were introduced starting with Boc-Orn-(Fmoc)-OH, and after usual peptide chain assembly followed by specific cleavage of the side chain Fmoc protection (10% diethylamine in DMF, 10 min) from the protected peptidyl-resin, the guanidino group was incorporated using 1*H*-pyrazole-1-carboxamidine hydrochloride in DMF/DIEA[30] in the last solid phase chemistry step before final HF deprotection and cleavage. Peptides containing *N,N'*-tetramethylguanidine side chains were prepared similarly from the appropriate N^{α}-Boc-N^{ω}-Fmoc-amino acid using HBTU/DIEA (10 equiv each) in minimal DMF (3 h, room temperature) to incorporate the tetramethylguanidino group.[31] Peptides with NH_2-acetyl and NH_2-propionyl side chains were also prepared by this strategy (i.e., via orthogonal Fmoc protection of side chain amines which, after deprotection, were acylated using standard reagents while still resin-bound). Completed protected peptidyl-resins were cleaved and deprotected using HF containing 5% anisole at 4 °C for 1 h. After HF removal *in vacuo*, the products were washed several times with diethyl ether and extracted with several portions into 20–30 mL of 5% HOAc in water. The entire solution of crude product thus obtained was purified by loading directly onto a preparative HPLC system with a C-18 column using a linear gradient of increasing acetonitrile in water containing 0.1% TFA for elution as previously detailed.[32] Fractions shown by HPLC to be >95% pure were pooled and lyophilized to provide, with a few exceptions, 25–45 mg of peptide products (ca. 30–50% overall yield) as white powder TFA salts that in general were >98% pure as determined by HPLC (YMC C_{18} column, detection at 215 and 280 nm using several gradients and solvent systems).

All peptides were found to have, within experimental variation (±5%), the expected composition as determined after hydrolysis[33] and amino acid analysis using the Pico Tag method,[34] which was also used to determine solution concentrations. Peptide identity was further confirmed by FAB mass spectral analysis with molecular ion peaks at $(M + H)^+$ and $(M - H)^-$ in negative ion mode, observed for all peptides. The ^1H NMR data for lead antagonist tetrapeptide **41** is representative: The spectrum (600 MHz) was obtained on a 3 mM solution of **41** in D_2O (pH 6.6) at 3 °C, and peak positions in δ (ppm) (peak positions for exchangeable amide NHs were determined with H_2O as solvent) are reported relative to residual HDO in solvent which resonates 5.02 ppm downfield from TSP. NMR: δ 0.89 (s, 3H, Leu CH_3), 0.955 (s, 3H, Leu CH_3), 1.48 (m, 1H, Leu γ CH), 1.635 (m, 3H, Leu γ CH, Leu β CH_2), 1.675 (m, 2H, Arg γ CH_2), 1.785 (m, 1H, Arg βCH_2), 1.88 (m, 1H, Arg βCH_2), 2.915 (m, 1H, Phe(Gn) βCH_2), 3.02 (m, 2H, Phe(fluoro) βCH_2), 3.13 (m, 1H, Phe(Gn) βCH_2), 3.20 (m, 2H, Arg δ CH_2), 4.25 (m, 1H, Arg α CH), 4.29 (m, 1H, Leu α CH), 4.59 (m, 1H, Phe(Gn) α CH), 4.63 (m, 1H, Phe(fluoro) α CH), 6.57 (d, 1H, J = 15.3 Hz, cinnamoyl α vinyl), 6.68 (d, 1H, C-terminal NH), 6.96 (d, 1H, C-terminal NH), 7.05 (m, 2H, aryl), 7.095 (m, 2H, aryl), 7.21 (m, 2H, aryl), 7.245 (m, 2H, aryl), 7.27 (m, 1H, Arg ϵ NH), 7.45 (d, 1H, cinnamoyl β vinyl overlapped with 7.49, m, 3H, cinnamoyl aryls), 7.66 (m, 2H, aryl), 8.22 (d, 1H, J = 6.8 Hz, Leu NH), 8.33 (d, 1H, J = 7.8 Hz, Phe(Gn) α NH), 8.42 (d, 1H, J = 7.0 Hz, Arg α NH), 8.50 (d, 1H, J = 7.6 Hz, Phe(fluoro) NH).

Biological Assays. Assays for human platelet aggregation activity,[21,35] platelet membrane GTPase activity,[21,23] and receptor binding assays using 20–25 nM [^{3}H] SFFLRR-NH_2 and a platelet membrane preparation were performed as described elsewhere.[24]

Abbreviations. Abbreviations for amino acids and nomenclature of peptide structures not specifically stated in the text follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* 1971, *247*, 997). Other abbreviations not given in the text are as follows: Boc = *tert*-butyloxycarbonyl; BOP = (1*H*-benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate; Cbz = benzyloxycarbonyl; DCC = *N,N'*-dicyclohexylcarbodiimide; DIEA = diisopropylethylamine; DMF = dimethylformamide; DVB = divinylbenzene; FAB = fast atom bombardment; Fmoc = [(9-fluorenylmethyl)oxy]carbonyl, HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMP = *N*-methylpyrrolidinone; SAR = structure–activity relationship; Tos = tosyl (*p*-toluenesulfonyl); TSP = (trimethylsilyl)[2,2,3,3-$^2H_4$]propionate.

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Pharmacology of Cannabinoid Receptor Ligands

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Abstract: Mammalian tissues contain at least two types of cannabinoid receptor, CB_1 and CB_2, both coupled to G proteins. CB_1 receptors are expressed mainly by neurones of the central and peripheral nervous system whereas CB_2 receptors occur in certain non-neuronal tissues, particularly in immune cells. The existence of endogenous ligands for cannabinoid receptors has also been demonstrated. The discovery of this 'endogenous cannabinoid system' has been paralleled by a renewed interest in possible therapeutic applications of cannabinoids, for example in the management of pain and in the suppression of muscle spasticity/spasm associated with multiple sclerosis or spinal cord injury. It has also prompted the development of a range of novel cannabinoid receptor ligands, including several that show marked selectivity for CB_1 or CB_2 receptors. This review summarizes current knowledge about the in vitro pharmacological properties of important CB_1 and CB_2 receptor ligands. Particular attention is paid to the binding properties of these ligands, to the efficacies of cannabinoid receptor agonists, as determined using cyclic AMP or [^{35}S]GTPγS binding assays, and to selected examples of how these pharmacological properties can be influenced by chemical structure. The in vitro pharmacological properties of ligands that can potently and selectively oppose the actions of CB_1 or CB_2 receptor agonists are also described. When administered by themselves, some of these ligands produce effects in certain tissue preparations that are opposite in direction to those produced by cannabinoid receptor agonists and the possibility that the ligands producing such 'inverse cannabimimetic effects' are inverse agonists rather than pure antagonists is discussed.

Introduction

An important recent advance in the field of cannabinoid research has been the discovery of the presence in mammalian tissues of at least two types of cannabinoid receptor, CB_1 and CB_2 [1, 2]. Both receptor types have been cloned, their predicted amino acid sequences showing a similarity of about 44% (35% to 82% within the individual transmembrane domains) [3]. CB_1 and CB_2 receptors are coupled through G_i or G_o proteins, negatively, to adenylate cyclase and to N- and P/Q-type calcium channels and positively to A-type and inwardly rectifying potassium channels and to mitogen activated protein kinase. Under certain conditions, CB_1 receptors may also act through G_s proteins to activate adenylate cyclase [4]. There is also evidence that CB_1 receptors may mobilize arachidonic acid and close 5-HT_3 receptor ion channels when activated [1]. In addition, cannabinoids can close sodium channels but whether this effect is receptor-mediated has yet to be established [1].

As to the distribution pattern of cannabinoid receptors, CB_1 receptors are found mainly on neurones in the brain, spinal cord and peripheral nervous system and an important function of these receptors seems to be modulation of the release of several different transmitters at particular central and peripheral loci [1, 2]. The distribution of CB_1 receptors within the CNS is consistent with the ability of psychotropic cannabinoids to impair cognition and memory and to alter the control of motor function and the perception of pain. Thus the cerebral cortex, hippocampus, caudate-putamen, substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus, cerebellum, central grey substance and dorsal horn of the spinal cord all contain significant numbers of CB_1 receptors [1, 2]. Although it is known that CB_2 receptors are expressed mainly by immune cells [1, 2], little is yet known about their physiological role.

The cloning of the CB_1 receptor in 1990 was followed by the demonstration in 1992 that mammalian tissues produce agonists for these receptors [1, 2]. The most important of these 'endocannabinoids' are arachidonoylethanolamide (anandamide) and 2-arachidonoyl glycerol (Fig. 1). Both these compounds are thought to serve as neuromodulators or neurotransmitters, there being evidence that they are synthesized within neurones, that they can undergo

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depolarization-dependent release from neurones and that once released they are rapidly removed from the extracellular space by tissue uptake and intracellular metabolism [5, 6]. Cannabinoid CB_1 and CB_2 receptors and endocannabinoids together constitute the 'endogenous cannabinoid system'.

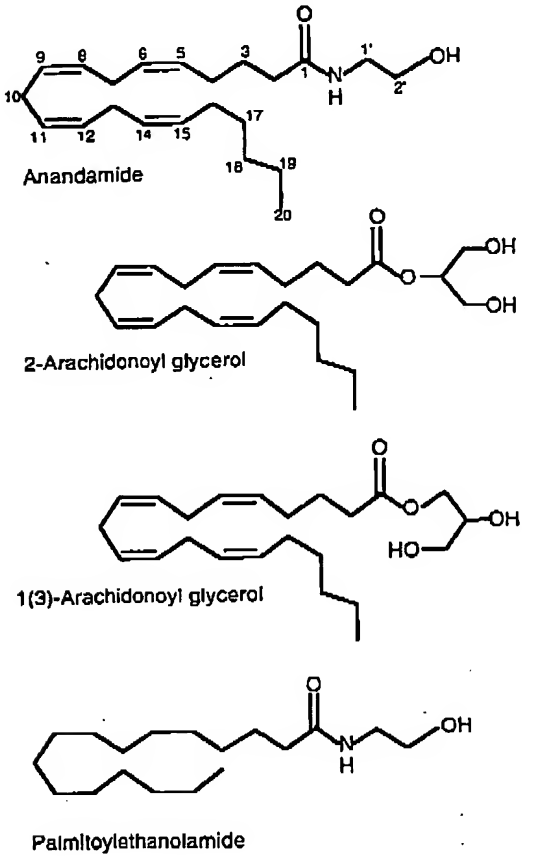


Fig. (1). Structures of anandamide, 1- and 2-arachidonoyl glycerol and palmitoylethanolamide.

The discovery of the endogenous cannabinoid system has prompted a search both for selective CB_1 and CB_2 receptor agonists and antagonists and for drugs that will selectively modulate the concentrations of endocannabinoids at their receptors, through effects on tissue uptake or enzymic hydrolysis. This review focuses on the pharmacological properties of just some of the many CB_1 and CB_2 receptor ligands that are now available. Included are Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychotropic constituent of cannabis, and nabilone (Fig. 2). Both these cannabinoids are licensed medicines [7]. Δ^9-THC, as the oral preparation dronabinol (Marinol), is available in the USA for the suppression of nausea and vomiting provoked by anticancer drugs and for the reversal, through appetite stimulation, of body weight loss experienced by AIDS patients. Nabilone (Cesamet), a synthetic analogue of Δ^9-THC that is also given by mouth, is licensed for use in the UK, again to suppress nausea and vomiting produced by cancer chemotherapy. Also included in this review is a range of compounds that are commonly used as experimental tools and/or that have therapeutic potential. Particular attention is paid to the affinities of these ligands for CB_1 and CB_2 receptors, to the extent that it has been possible to develop agents with selectivity for CB_1 or CB_2 receptors and to the relative efficacies of agonists at CB_1 and CB_2 receptors. The question of whether so called cannabinoid receptor antagonists are really inverse agonists is also addressed. Although no attempt has been made to give a complete account of what is now known about the structural features of compounds that determine cannabinoid receptor affinity or efficacy, mention is made of a few observations that have provided important insights into such structure-activity relationships. The review begins with a brief account of the various in vitro bioassay systems that are now most widely used to characterize cannabinoid receptor ligands.

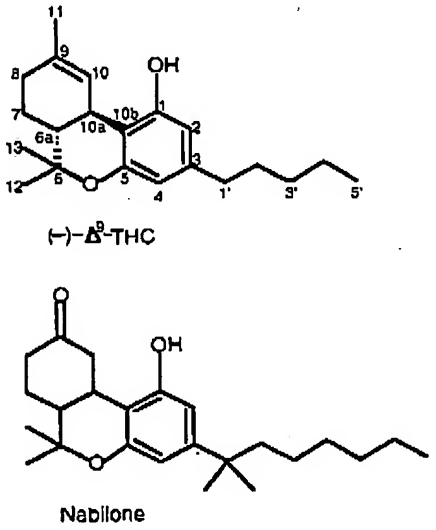


Fig. (2). Structures of (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC) and nabilone.

Bioassay Systems for Determining the CB_1 and CB_2 Binding Properties and Pharmacological Activity of Cannabinoid Receptor Ligands

In vivo assays have played an important part in characterizing the pharmacology and structure activity relationships of ligands for CB_1 receptors [8] and remain important for determining the acute and chronic pharmacological and toxicological profiles of cannabinoids in the whole organism. However, for establishing the pharmacological properties of any novel cannabinoid, particularly its CB_1 and CB_2 receptor affinity and efficacy, the starting point now must be the

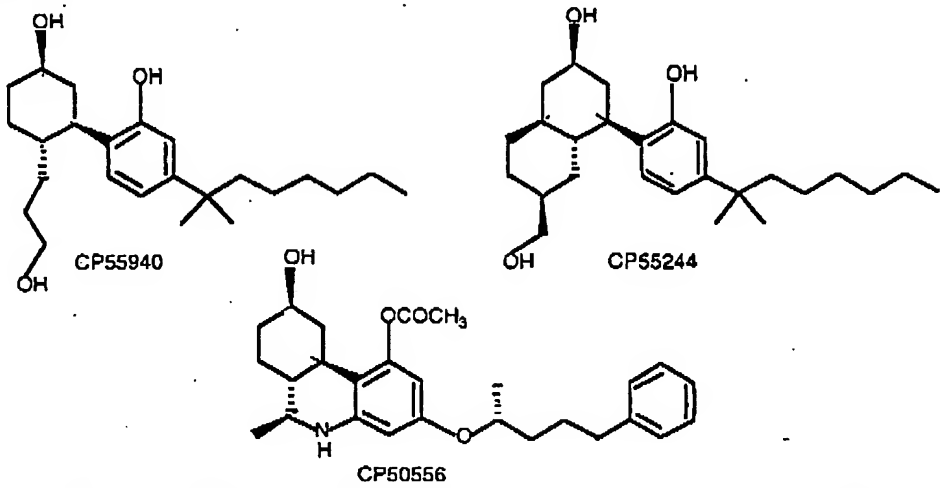


Fig. (3). Structures of CP55940, CP55244 and CP50556 (L-nantradol). The (+)-enantiomer of CP55940 is CP56667.

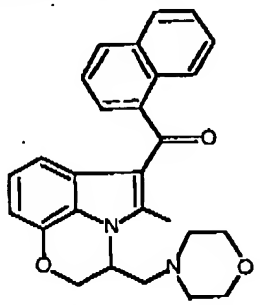
small set of in vitro 'gold standard' bioassays described below.

Binding Assays with Radiolabelled Cannabinoid Receptor Probes

Several cannabinoid receptor ligands have been radiolabelled with tritium and these have played a vital part both in determining the affinities of unlabelled cannabinoids for CB_1 and CB_2 receptors in displacement assays and in establishing the tissue distribution of cannabinoid receptors [1]. Commercially available radiolabelled cannabinoid receptor ligands are [^{3}H]SR141716A, which is CB1-selective, and [^{3}H]CP55940, [^{3}H](+)-WIN55212 and [^{3}H]HU-243, which bind more less equally well to CB_1 and CB_2 receptors (Table 1). The structures of CP55940, WIN55212, SR141716A and [^{3}H]HU-243 are shown in Figs. 3 to 6.

Table 1. Dissociation Constant (K_D) Values of Radiolabelled Ligands at Specific CB_1 and CB_2 Binding Sites in Brain or Transfected Cell Membranes

Radioligand	Source of membranes	K_D (nM)	Reference
[^{3}H]SR141716A	Guinea-pig forebrain	1.24	[26]
	Rat cerebellum (10 brain areas studied)	0.19	[22]
	Rat cerebellum	0.50	[19]
	Rat cerebellum	0.59	[17]
	Rat cerebellum	0.61	[20]
	Rat brain minus cerebellum	0.61	[15]
	Primary culture of rat cortical neurones	0.76	[18]
	Rat brain	1.20	[25]
[^{123}I]AM251	Rodent cerebellum (3 brain areas studied)	0.25	[21]
[^{3}H](+)-WIN55212	Guinea-pig forebrain	2.34	[26]
	Rat cerebellum	1.89	[10]
	Rat cerebellum (10 brain areas studied)	4.67	[22]
	Rat cerebellum	8.60	[23]
	Rat CB_1 AtT-20 cells	2.60	[13]
	Human CB_1 293 cells	11.9	[16]
	Human CB_1 CHO cells	16.2	[24]
	Human CB_2 COS cells	2.10	[12]
	Human CB_2 COS cells	3.70	[11]
	Human CB_2 CHO cells	3.80	[12]
[^{3}H]HU-243	Rat brain minus brain stem	0.045	[9]
	Human CB_2 CHO cells	0.061	[14]
[^{3}H]CP55940	Human CB_1 cells	0.4 to 3.3	[1]
	Rat CB_1 cells	4.00	[1]
	Rat brain	0.07 to 2.3	[1]
	Mouse brain	3.4	[1]
	Human CB_2 cells	0.2 to 7.37	[1]
	Mouse CB_2 cells	0.394	[1]



WIN55212

Fig. (4). Structure of WIN55212.

The most widely used radiolabelled cannabinoid is [^{3}H]CP55940. As this has the same affinity for CB_1 and CB_2 receptors, results obtained from displacement assays using this radioligand will only yield useful information about the binding properties of unlabelled compounds if they are performed with membranes that are known to contain either CB_1 or CB_2 receptors but not both. Ideally, these membranes should be obtained from CB_1 or CB_2 transfected cells. An alternative is to use tissue that expresses CB_1 or CB_2 receptors naturally, for example brain tissue for CB_1 receptors and spleen tissue for CB_2 receptors. Although often used, these tissues are less satisfactory for displacement binding assays than transfected cells. Thus although brain tissue is largely populated with CB_1 receptors, some CB_2 receptors may also be present, on neurones and astrocytes [27, 28]. Similarly, although most cannabinoid receptors in the spleen are CB_2, some CB_1 receptors are also present in this tissue [1]. There is also the possibility that brain and/or spleen express types of cannabinoid receptor yet to be identified. Indeed, there is already some evidence, albeit still inconclusive, that mammalian brain, spinal cord and peripheral nervous system can express additional cannabinoid receptor types/subtypes [28, 29]. Binding assays performed with membranes from brain, spleen or other cannabinoid-receptor containing tissues using well-characterized cannabinoid receptor ligands provide a powerful means of searching for novel cannabinoid receptor types/subtypes. Another commercially available radiolabelled cannabinoid receptor probe is [^{3}H]anandamide. However, this tends to be used in experiments designed to explore the biochemistry and pharmacology of anandamide tissue uptake and metabolism rather than in receptor binding assays. The CB_2-selective ligand, [^{3}H]SR144528 has also been synthesized but is not yet generally available. Representative CB_1/CB_2 K_D values of [^{3}H]SR141716A, [^{3}H]CP55940, [^{3}H](+)-WIN55212 and [^{3}H]HU-243 are shown in Table 1 whilst values for the relative CB_1/CB_2 affinities of unlabelled SR141716A, CP55940, (+)-WIN55212 and anandamide are given in Table 2.

Table 2. The Abilities of Certain Ligands to Bind to CB_1 and CB_2 Receptors

Displacing Ligand	Radioligand	CB_1 membranes	CB_2 membranes	CB_1 K_i (nM)	CB_2 K_i (nM)	Affinity ratio CB_1/CB_2	Affinity ratio CB_2/CB_1	Reference
CB_1 selective								
ACEA (Fig. 11)	[^{3}H]CP55940	Rat cerebellum	Rat spleen	1.4*	>2000*	>1428	<0.0007	[46]
SR141716A (Fig. 5)	[^{3}H]CP55940	hCB_1 in L cells	hCB_2 in CHO cells	11.8	13200	1118.64	0.00089	[42]
SR141716A	[^{3}H]CP55940	*hCB_1* in L cells	hCB_2 in AtT-20 cells	11.8	973	82.46	0.012	[32]
SR141716A	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	12.3	702	57.07	0.018	[37]
SR141716A	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	5.6	>1000	>178.57	<0.0056	[30]
SR141716A	[^{3}H]CP55940	Rat brain†	Rat spleen	1.98	>1000	>505.05	<0.002	[30]
ACPA (Fig. 11)	[^{3}H]CP55940	Rat cerebellum	Rat spleen	2.2*	715*	325.00	0.0031	[46]
LY320135 (Fig. 14)	[^{3}H]CP55940	hCB_1 in L cells	hCB_2 in CHO cells	141	14900	105.67	0.0095	[42]
Methanandamide (Fig. 11)	[^{3}H]CP55940	Rat forebrain	Mouse spleen	17.9*	868*	48.49	0.021	[45]
Methanandamide	[^{3}H]CP55940	Rat forebrain	Mouse spleen	20*	815	40.75	0.025	[40]
O-585 (Fig. 11)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	8.6*	324*	37.67	0.027	[37]
O-689 (Fig. 11)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	5.7*	132*	23.16	0.043	[37]
Similar affinities for CB_1 and CB_2 receptors								
Anandamide (Fig. 1)	[^{3}H]CP55940	Rat forebrain	Mouse spleen	61*	1930*	31.64	0.032	[45]

(Table 2), contd.....

Displacing Ligand	Radioligand	CB_1 membranes	CB_2 membranes	CB_1 K_i (nM)	CB_2 K_i (nM)	Affinity ratio CB_1/CB_2	Affinity ratio CB_2/CB_1	Reference
Anandamide	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	89*	371*	4.17	0.24	[37]
Anandamide	[^{3}H]CP55940	hCB_1 in L cells	hCB_2 in AtT-20 cells	543	1940	3.57	0.28	[32]
Anandamide	[^{3}H]CP55940	Rat cerebellum	Rat spleen	71.7*	279*	3.89	0.26	[46]
Anandamide	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in COS cells	252	581	2.31	0.43	[31]
AM404 (Fig. 12)	[^{3}H]CP55940	Rat forebrain	Mouse spleen	1760*	13000	7.39	0.14	[40]
CT-3 (Fig. 7)	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in CHO cells	32.3	170.5	5.28	0.19	[41]
2-AG (Fig. 1)	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in COS cells	472	1400	2.97	0.34	[31]
2-AG	[^{3}H]HU-243	CB_1 in COS cells	CB_2 in COS cells	58.3	145	2.49	0.40	[44]
11-OH-CBN-DMH (Fig. 8)	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in CHO cells	0.1	0.2	2.00	0.50	[41]
HU-210 (Fig. 6)	[^{3}H]CP55940	hCB_1 in L cells	hCB_2 in AtT-20 cells	0.0608	0.524	8.62	0.12	[32]
HU-210	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in CHO cells	0.1	0.17	1.70	0.59	[41]
HU-210	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	0.73	0.22	0.30	3.32	[37]
HU-211 (Fig. 6)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	1990	>10,000	>5.0	<0.2	[37]
O-1184 (Fig. 9)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	5.25	7.41	1.41	0.71	[49]
Δ^9-THC (Fig. 2)	[^{3}H]CP55940	hCB_1 in L cells	hCB_2 in AtT-20 cells	53.3	75.3	1.41	0.71	[32]
Δ^9-THC	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in COS cells	39.5	40	1.01	0.99	[34]
Δ^9-THC	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	40.7	35.4	0.89	1.12	[37]
Δ^9-THC	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in CHO cells	80.3	32.2	0.40	2.49	[41]
Δ^9-THC	[^{3}H]CP55940	Rat brain†	Rat spleen	35.3	3.9	0.11	9.05	[30]
Nabilone (Fig. 2)	[^{3}H]CP55940	hCB_1	hCB_2	1.84	2.19	1.19	0.84	[33]
Δ^8-THC (Fig. 7)	[^{3}H]CP55940	Rat forebrain	Mouse spleen	47.6	39.3	0.83	1.21	[36]
Δ^9-THC-DMH (Fig. 7)	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in CHO cells	0.241	0.199	0.83	1.21	[41]
CBN-DMH (Fig. 8)	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in CHO cells	2	1.5	0.75	1.33	[41]
CP55940 (Fig. 3)	[^{3}H]CP55940	hCB_1 *in CHO cells*	hCB_2 in CHO cells	5	1.8	0.36	2.78	[47]
CP55940	[^{3}H]CP55940	hCB_1 in L cells	hCB_2 in AtT-20 cells	3.72	2.55	0.69	1.46	[32]
CP55940	[^{3}H]CP55940	Rat brain†	Rat spleen	1.37	1.37	1.0	1.0	[30]
CP55940	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	0.58	0.69	1.19	0.84	[37]
CP55940	[^{3}H]CP55940	Rat cerebellum	Rat spleen	0.50*	2.80*	5.60	0.18	[46]
11-OH-CBN (Fig. 8)	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in CHO cells	38	26.6	0.70	1.43	[41]
Cannabidiol (Fig. 8)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	4350	2860	0.66	1.52	[37]
Cannabinol (Fig. 8)	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in CHO cells	211.2	126.4	0.60	1.67	[41]
Cannabinol	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	308	96.3	0.31	3.20	[37]
Cannabinol	[^{3}H]CP55940	hCB_1 in L cells	hCB_2 in AtT-20 cells	1130	301	0.27	3.75	[32]
CP56667 (Fig. 3)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	61.7	23.6	0.38	2.61	[37]
11-OH-Δ^8-THC (Fig. 7)	[^{3}H]HU-243	rCB_1 in COS cells	hCB_2 in CHO cells	25.8	7.4	0.29	3.49	[41]
1-deoxy-Δ^8-THC-DMH (Fig. 10)	[^{3}H]CP55940	Rat cerebral cortex	hCB_2 in CHO cells	23	2.9	0.13	7.93	[38]

(Table 2). contd.....

Displacing Ligand	Radioligand	CB_1 membranes	CB_2 membranes	CB_1 K_i (nM)	CB_2 K_i (nM)	Affinity ratio CB_1/CB_2	Affinity ratio CB_2/CB_1	Reference
CB_2 selective								
(+)-WIN55212 (Fig. 4)	[^{3}H]CP55940	Rat brain†	Rat spleen	9.94	16.2	1.63	0.61	[30]
(+)-WIN55212	[^{3}H]CP55940	Rat cerebellum	Rat spleen	4.4*	1.2*	0.27	3.67	[46]
(+)-WIN55212	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	1.89	0.28	0.15	6.75	[37]
(+)-WIN55212	[^{3}H]CP55940	hCB_1 in L cells	hCB_2 in AtT-20 cells	62.3	3.3	0.053	18.88	[32]
(+)-WIN55212	[^{3}H]CP55940	hCB_1 in COS cells	hCB_2 in COS cells	123	4.1	0.033	30.00	[35]
JWH-015 (Fig. 13)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	383	13.8	0.036	27.75	[37]
JWH-051 (Fig. 10)	[^{3}H]CP55940	Rat cerebral cortex	hCB_2 in CHO cells	1.2	0.032	0.027	37.50	[38]
L768242 (Fig. 13)	[^{3}H]CP55940	hCB_1	hCB_2	1917	12	0.0063	159.75	[39]
JWH-139 (Fig. 10)	[^{3}H]CP55940	Rat cerebral cortex	hCB_2 in CHO cells	2290	14	0.0061	163.57	[48]
AM630 (Fig. 13)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	5152	31.2	0.0061	165.13	[47]
JWH-133 Fig. 10)	[^{3}H]CP55940	Rat cerebral cortex	hCB_2 in CHO cells	677	3.4	0.005	199.12	[48]
1-deoxy-Δ^8-THC (Fig. 10)	[^{3}H]CP55940	Rat cerebral cortex	hCB_2 in CHO cells	8770	32	0.0037	274.1	[48]
L759633 (Fig. 10)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	1043	6.4	0.0061	162.97	[47]
L759633	[^{3}H]CP55940	hCB_1	hCB_2	15850	20	0.0013	792.5	[33]
L759656 (Fig. 10)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	4888	11.8	0.0024	414.24	[47]
L759656	[^{3}H]CP55940	hCB_1	hCB_2	>20000	19.4	<0.00097	>1000	[33]
SR144528 (Fig. 5)	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	437	0.60	0.0014	728.33	[43]
SR144528	[^{3}H]CP55940	Rat brain†	Rat spleen	305	0.30	0.00098	1016.67	[43]
SR144528	[^{3}H]CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	>10000	5.6	<0.00056	>1785	[47]

†Minus cerebellum. *With PMSF 50 to 300 μM.
CP56667 is the (+)-enantiomer of CP55940. HU-211 is the (+)-enantiomer of HU-210.
h, human; r, rat; ACEA, arachidonoyl-2-chloroethylamide; ACPA, arachidonoylcyclopropylamide; 2-AG, 2-arachidonoyl glycerol, CBN, cannabinol; DMH, dimethylheptyl; THC, tetrahydrocannabinol.

Three other radiolabelled ligands have been developed as potential probes for human single photon emission computed tomography (SPECT) or positron emission tomography (PET) studies, all analogues of SR141716A (Fig. 5). One of these is an [^{18}F]-labelled analogue, SR144385 [50]. Another is [^{123}I]AM251, in which the 4'-Cl of the monochlorophenyl group of SR141716A is replaced by 4'-I [21, 51] (Table 1). The third of these radiolabelled ligands is [^{123}I]AM281 in which the piperidine ring of SR141716A is replaced by a more polar morpholino group [52]. AM281antagonizes (+)-WIN55212-induced inhibition of evoked acetylcholine release in rat hippocampal slices with a similar potency to SR141716A [53]. Like SR141716A (see below), it also enhances evoked acetylcholine release in rat hippocampal slices when administered alone [53].

Functional In vitro Assays

Two in vitro bioassay systems have yielded most information about the efficacy of ligands at CB_1 and CB_2 receptors. One of these measures net agonist-stimulated [^{35}S]GTPγS binding to G protein (Table 3). It relies on one of the events triggered by the occupation of CB_1 and CB_2 receptors by agonist molecules: increased G protein affinity for GTP (and [^{35}S]GTPγS). The occupation of a CB_1 or CB_2 receptor by an agonist causes the α and βγ subunits of the heterotrimeric G protein to which it is coupled to dissociate and shifts the α subunit from a state in which it has higher affinity for GDP than GTP to one in which it has lower affinity for GDP than GTP. Once dissociated, the α and βγ subunits both act upon effectors until the GTP bound to the α subunit is dephosphorylated to GDP by the action of α subunit GTPase. The α and βγ subunit re-

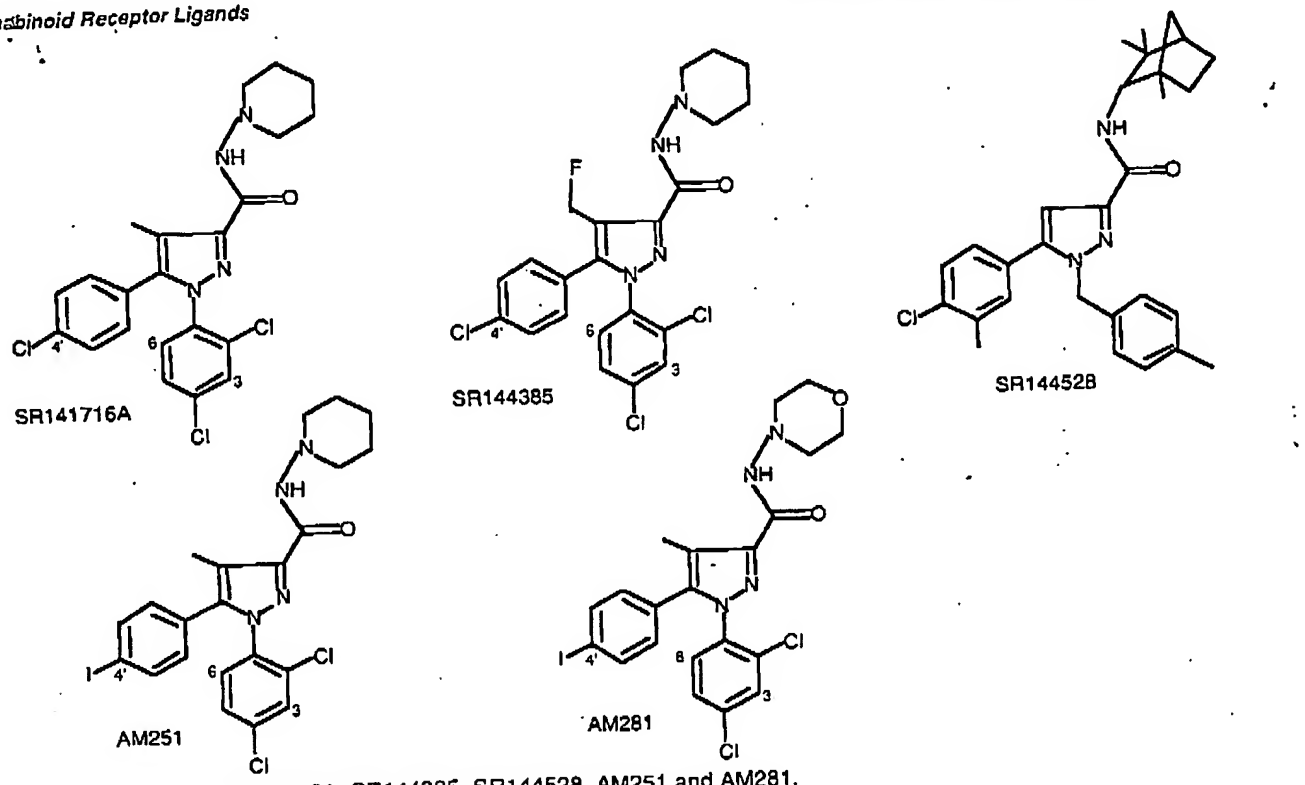


Fig. (5). Structures of SR141716A, SR144385, SR144528, AM251 and AM281.

associate and the reconstituted G protein is then ready for further activation by agonist. For an agonist to produce sufficient stimulation of [^{35}S]GTPγS membrane binding, it is important that the binding of [^{35}S]GTPγS that occurs in the absence of added agonist is minimized. This is achieved by adding high amounts of GDP and sodium chloride [55, 67, 69]. GDP decreases agonist-stimulated as well as basal binding of [^{35}S]GTPγS. However, it is the basal binding that it inhibits the more, the overall result being the production by GDP of concentration-related increases in net agonist-stimulated [^{35}S]GTPγS binding [67]. The amount by which GDP concentrations can be increased to enhance net agonist-stimulated [^{35}S]GTPγS binding is limited by the concentration-related inhibitory effect GDP has on absolute levels of basal and agonist-stimulated of binding. Thus, when GDP concentrations are progressively raised, a point is eventually reached at which [^{35}S]GTPγS binding has fallen to a level that is too low to be measured reproducibly [55].

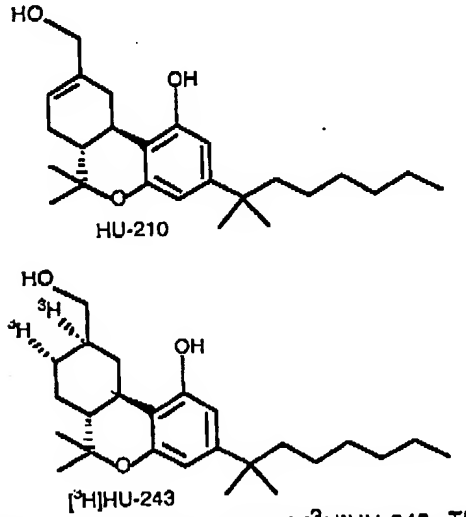


Fig. (6). Structures of HU-210 and [^{3}H]HU-243. The (+)-enantiomer of HU-210 is HU-211.

The other bioassay system exploits the negative coupling of CB_1 and CB_2 receptors to adenylate cyclase, the measured response usually being agonist-induced inhibition of forskolin-stimulated cyclic AMP production in cannabinoid receptor-containing tissue preparations (Tables 4 and 5). Although CB_1 or CB_2 transfected cells are now most frequently used to characterize novel cannabinoid receptor ligands with this assay, tissues that express CB_1 or CB_2 naturally have also been used. These include brain synaptosomes and primary cultures of central neurones or spleen cells (see above). Some information about the efficacy of ligands for CB_1 receptors has also been gleaned from a third bioassay that exploits the negative coupling of CB_1 (but not CB_2) receptors to N- and P/Q-type calcium channels [1]. In this case the measured response is agonist-induced inhibition of depolarization-stimulated inward calcium current in CB_1 receptor-containing tissue preparations (Table 6). However, this system is less suitable for routine use.

Table 3. Stimulatory Effect of Certain Cannabinoid Receptor Ligands on GTPγS Binding by Tissue or Cultured Cell Membranes Expressing Cannabinoid CB_1 or CB_2 Receptors

Preparation	Experimental Conditions	EC_{50} values (nM) and maximal degree of stimulation over basal (%)												Ref.
		CP559	CP552	CP505	THC	HU	(+)-WIN	(-)-WIN	AEA	Met	O-689	O-1064	CBN	
Human CB_1-transfected CHO cell membranes	0.1 nM [^{35}S]GTPγS; 50 µM GDP; 100 mM NaCl; 3 mM $MgCl_2$; 30 µg protein; 0.2 mM EGTA; 0.1% BSA; 60 min at 30°C (0.2 ml assay)	– ≈40% by 20 nM	–	–	–	–	–	–	–	–	–	–	–	[59]
Human CB_1-transfected CHO cell membranes	0.1 nM [^{35}S]GTPγS; 50 µM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 0.1% tissue; 1 mM EDTA; 0.25% BSA; 90 min at 30°C (1 ml assay)	–	–	–	–	–	473* 76%	–	–	–	–	–	–	[60]
Human CB_1-transfected CHO cell membranes	0.1 nM [^{35}S]GTPγS; 50 µM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 0.1% tissue; 1 mM EDTA; 0.25% BSA; 90 min at 30°C (1 ml assay)	–	–	–	–	–	360* 77.8%	–	–	–	–	–	–	[63]
Human CB_1-transfected CHO cell membranes	1 nM [^{35}S]GTPγS; 320 µM GDP; 100 mM NaCl; 32 mM $MgCl_2$; 5 µg protein; 60 min at 37°C (0.25 ml assay)	16.6 100%	–	–	–	–	616.6 92%	–	–	–	–	–	>10 µM ~5%	[66]
Rat striatum membranes	0.05 nM [^{35}S]GTPγS; 20 µM GDP; 100 mM NaCl; 3 mM $MgCl_2$; 4 µg protein; 0.2 mM EGTA; 0.1 mg BSA; 60 min at 30°C (1 ml assay)	–	–	–	–	–	20 125%	–	–	–	–	–	–	[54]
Rat cerebellum membranes	0.05 nM [^{35}S]GTPγS; 50 µM GDP; 100 mM NaCl; 3 mM $MgCl_2$; 18 µg protein; 0.2 mM EGTA; 0.1% BSA; 60 min at 30°C (1 ml assay)	100 140%	–	600 167%	– 0%	–	180 162%	>10000 3%**	≥5100* –	–	–	–	–	[55]
Rat cerebellum membranes	0.05 nM [^{35}S]GTPγS; 20 µM GDP; 100 mM NaCl; 3 mM $MgCl_2$; 15 µg protein; 0.2 mM EGTA; 0.1 mg BSA; 60 min at 30°C (1 ml assay)	–	–	–	– 20%	–	120 270%	–	–	–	–	–	–	[19]
Rat cerebellum membranes	0.2 nM [^{35}S]GTPγS; 100 µM GDP; 1 mM $MgCl_2$; 15 µg protein; 1 mM EGTA; 0.25 mg BSA; 90 min at 30°C (0.5 ml assay)	9 100%	–	–	530 54%	–	89 75%	>10000	540* 73%	180 104%	–	–	170 30%	[62]
Rat cerebellum membranes	0.05 nM [^{35}S]GTPγS; 100 (or 10#) µM GDP; 150 mM NaCl; 9 mM $MgCl_2$ 10 µg protein; 0.2 mM EGTA; 1% BSA; 60 min (HU) or 30 min at 30°C (0.5 ml assay)	17.6 114% 61%#	0.47 165%	–	– 0% 51%#	0.55 140%	151 156% 89%#	–	–* 0%	–	25.4 97%	245 80%	– 0%	[64]
Rat cerebellum membranes	0.2 nM [^{35}S]GTPγS; 100 µM GDP; 1 mM $MgCl_2$; 15 µg protein; 1 mM EGTA; 0.25 mg BSA; 90 min at 30°C (0.5 ml assay)	53 87%	–	–	216 54%	–	–	–	–	–	–	–	187 24%	[65]
Rat cerebellum membranes	0.05 nM [^{35}S]GTPγS; 30 µM GDP; 100 mM NaCl; 4-15 µg protein; 0.1% BSA; 120 min at 30°C (1 ml assay)	8.8 55%†	–	8 117%†	87 25%†	–	180 125%†	–	390* 82%†	320 79%†	–	–	–	[67]
Rat cerebellum membranes	0.65 nM [^{35}S]GTPγS; 10 µM GDP; 150 mM NaCl; 5 µg protein; 0.1% BSA; 30 min at 37°C	31 100%	–	–	–	–	120 117%	–	276* 66%	–	–	–	–	[46]
Rat cerebellum membranes	0.05 nM [^{35}S]GTPγS; 50 µM GDP; 100 mM NaCl; 5 mM $MgCl_2$; 40-50 µg protein; 0.1% BSA; 60 min at 30°C	100 145%	–	–	–	–	180 150%	–	–	330 148%	–	–	–	[45]

(Table 3). contd.....

Preparation	Experimental Conditions	EC_{50} values (nM) and maximal degree of stimulation over basal (%)												
		CP559	CP552	CP505	THC	HU	(+)-WIN	(-)-WIN	AEA	Met	O-689	O-1064	CBN	Ref.
Rat (a) cerebellum (b) amygdala (c) hypothalamus membranes	0.05 nM [^{35}S]GTPγS; 30 μM GDP; 100 mM NaCl; 3 mM $MgCl_2$; 10-20 μg protein; 0.2 mM EGTA; 0.1% BSA at 30°C (1 ml assay)	-	-	-	-	-	(a) 158 (b) 84 (c) 131	-	-	-	-	-	-	[22]
Mouse whole brain membranes	0.1 nM [^{35}S]GTPγS; 50 μM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 1 mM EDTA; 90 min at 30°C (1 ml assay)	-	-	-	-	-	~320*	-	-	-	-	-	-	[56]
Mouse whole brain membranes	0.1 nM [^{35}S]GTPγS; 50 μM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 1 mM EDTA; 0.25% BSA; 90 min at 30°C	-	-	-	59* ~30%	-	347* ~150%	-	-	-	-	-	-	[57]
Mouse whole brain membranes	0.1 nM [^{35}S]GTPγS; 50 μM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 1 mM EDTA; 0.25% BSA; 90 min at 30°C (1 ml assay)	61.7* 136%	-	-	70.9* 37%	2.26* 123%	-	-	846* 99%	-	-	-	-	[61]
Mouse whole brain membranes	0.1 nM [^{35}S]GTPγS; 100 μM GDP; 100 mM NaCl; 3 mM $MgCl_2$; 50 μg protein; 0.2 mM EDTA; 50 μg BSA; 60 min at 30°C (0.5 ml assay)	200 75%	-	-	-	-	-	-	-	-	-	-	-	[68]
Guinea-pig whole brain membranes	0.1 nM [^{35}S]GTPγS; 50 μM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 1 mM EDTA; 90 min at 30°C (1 ml assay)	-	-	-	-	-	2980*	-	-	-	-	-	-	[59]
Human CB_2 transfected CHO cell membranes	1 nM [^{35}S]GTPγS; 320 μM GDP; 100 mM NaCl; 32 mM $MgCl_2$; 5 μg protein; 60 min at 37°C (0.25 ml assay)	2.75 42%	-	-	-	-	2.24 27%	-	-	-	-	-	575.4 -53%	[66]

* In the presence of 50 to 150 μM phenylmethylsulphonyl fluoride
** at 10 μM (-)-WIN55212
† Recalculated from normalized data, using 125% as the value for (+)-WIN55212 [67].
AEA, anandamide (Fig. 1); BSA, bovine serum albumin; CBN, cannabinol (Fig. 6); CHO, Chinese hamster ovary; CP559, CP55940 (Fig. 3); CP552, CP55244 (Fig. 3); O-689, fluoromethanandamide (Fig. 11); HU, HU-210 (Fig. 6); CP505, CP50556 (Fig. 3); Met, methanandamide (Fig. 11); O-1064, 2,16,16-trimethyl-all-cis-5,8,11,14-docosatetraenoyl-2'-fluoroethanolamide (Fig. 11); THC, Δ^9-THC (Fig. 2); WIN, WIN55212 (Fig. 4).

The [^{35}S]GTPγS assay is the least sensitive of the three functional bioassays described in this section (Tables 3 to 6). Presumably this is because the cannabinoid receptor signal is less amplified in this assay, adenylate cyclase and calcium channels both being located further along the signalling cascade than G protein. The [^{35}S]GTPγS assay also differs from the other two assays in providing a total measure of G protein-mediated cannabinoid receptor activation rather than a measure of the activation of just one particular cannabinoid receptor effector mechanism. This assay should, therefore, be independent of any variations that may exist between tissues in the relative contribution made by different G protein-coupled effector mechanisms.

A measure of the relative efficacies of cannabinoid receptor agonists can be obtained in all three assay systems by constructing log dose response curves and then comparing the size of responses to maximal doses (E_{max} values). Indeed, there is usually no other way of obtaining an indication of the relative efficacies of cannabinoid receptor agonists from the existing published data. One exception to this is to be found in a paper by Burkey *et al.* [61] which contains relative efficacy values at CB_1 receptors for CP55940, 11-OH-Δ^8-THC-dimethylheptyl (HU-210), anandamide and Δ^9-THC estimated from [^{35}S]GTPγS binding data. It is noteworthy that the maximal effects of what appear to be full cannabinoid receptor agonists on forskolin-stimulated cyclic AMP production and on depolarization-stimulated inward calcium current usually fall well short of complete inhibition (Tables 4 to 6).

In the literature, and hence also in Tables 3 to 6, the potency of an agonist is usually expressed as the concentration (EC_{50}) of that agonist lying at the mid point of its log concentration-response curve. Caution should therefore be exercised in using these EC_{50} values to calculate relative potency values as (a) not all cannabinoid receptor agonists elicit the same maximal response in any given assay system (Tables 3 to 6) and (b) relative potency values should be calculated by comparing the concentrations of different drugs that elicit the same sized response. This point is well illustrated by the data of Burkey *et al.* [57] who reported

that Δ^9-THC has a 5.9 fold lower EC_{50} than (+)-WIN55212 in the [^{35}S]GTPγS binding assay. The EC_{50} of Δ^9-THC (59 nM) produced only about 15% stimulation of [^{35}S]GTPγS binding whereas the EC_{50} of (+)-WIN55212 (347 nM) produced about 75% stimulation. (+)-WIN55212 matched the half-maximal response to Δ^9-THC (15% stimulation) at a concentration of about 25 nM, showing it to be more than twice as potent as Δ^9-THC in this assay.

Table 4. Inhibitory Effect of Certain Cannabinoids on Cyclic AMP Production by Cultured Cells Expressing Cannabinoid CB_1 Receptors or by Brain Tissue Stimulated by Forskolin (FSK), Secretin, Prostaglandin E_1, Prostacyclin or Isoprenaline

Preparation	Mode of cyclic AMP stimulation	Inhibitory EC_{50} values (nM) and maximal degree of inhibition (%)												
		CP559	CP552	CP505	THC	HU	Nab	(+)-WIN	(−)-WIN	AEA	2-AG	PEA	CBN	Ref.
Human CB_1-transfected CHO cells	0.5 μM FSK	0.99 80%	0.128 80%	–	13 ~40%	–	–	–	–	–	–	–	–	[77]
Human CB_1-transfected CHO cells	0.5 μM FSK	–	–	–	–	–	–	–	–	180 ~100%	–	>1 mM 0% at 1 mM	–	[81]
Human CB_1-transfected CHO cells	FSK	1.27	–	–	–	–	–	–	–	–	–	–	–	[30]
Human CB_1-transfected CHO cells	FSK	1.83	–	–	16.5	0.187	–	24	–	322	–	–	>1 μM 0% at 1 μM	[32]
Human CB_1-transfected CHO cells	FSK	1.2	–	–	10	–	–	14	–	–	–	–	–	[89]
Human CB_1-transfected 293 cells	FSK	3.1 ~45%	–	–	–	0.19 ~55%	–	7.8 ~40%	>1 μM 0% at 1 μM	81.8* ~75%	–	–	–	[16]
Human CB_1-transfected CHO cells	3 μM FSK	0.16 ~75%	–	–	2.25 ~75%	–	–	17.95 ~75%	–	–	–	–	–	[59]
Human CB_1-transfected CHO cells	3 μM FSK	2 ~83%	–	–	10.7 ~80%	–	–	44.3 ~60%	–	108.5 ~29%	–	–	–	[91]
Human CB_1-transfected CHO cells	1 μM FSK	4 62%	–	–	–	–	–	31 75.1%	–	–	–	–	–	[24]
Human CB_1-transfected CHO cells	3 μM FSK	2 75%	–	–	–	–	–	–	–	–	–	–	–	[43]
Human CB_1-transfected 293 cells	0.5 μM FSK	4.17 63%	–	–	–	–	–	1.64 63%	–	–	–	–	–	[93]
CB_1-transfected COS cells	1 μM FSK	–	–	–	–	–	–	–	–	–	428* ~80%	–	–	[44]
Rat CB_1-transfected CHO cells	0.5 μM FSK	0.87 ~60%	–	–	13.5 ~40%	–	16.6 ~60%	–	–	–	–	–	– ~12% at 1 μM	[75]
Rat CB_1-transfected CHO cells	–	1.8	0.2	2.4	55	0.02	3.1	34	>2000	–	–	–	–	[78]
Rat CB_1-transfected CHO cells	1 μM FSK	–	–	–	9.1 ~45%	0.9 50-55%	–	–	–	207 35-40%	–	>10 μM 0% at 0.1nM to 10 μM	–	[80]
Rat CB_1-transfected CHO cells	PGE_1	–	–	–	9.2 ~45%	–	–	–	–	100.5 ~60%	–	–	–	[98]
Rat CB_1-transfected COS cells	1 μM FSK	–	–	–	13 62%	–	–	–	–	–	–	–	–	[34]
Rat CB_1-transfected COS cells	1 μM FSK	–	–	–	11	0.035	–	–	–	–	–	–	120	[41]
Human U373 MG astrocytoma cells	5 μM FSK	0.8 ~100%	–	–	–	–	–	32 ~100%	–	–	–	–	–	[86]
Mouse N18TG2 cell membranes	1 μM FSK#	–	–	~100 ~32%	~220 ~29%	–	–	–	–	–	–	–	~500 ~9%	[70]
Mouse N18TG2 cell membranes	100 μM secretin	–	–	–	530 ~30%	–	–	–	–	–	–	–	1400 ~14%	[72]
Mouse N18TG2 cell membranes	0.5 μM secretin	25 39%	5 39%	100	430	–	–	–	–	–	–	–	–	[73]
Mouse N18TG2 cell membranes	0.5 μM secretin	–	–	–	–	7.2 (22%)	–	–	–	–	–	–	–	[74]
Mouse N18TG2 cells	1 μM FSK	–	–	–	–	2.9 ~60%	–	–	–	540 ~40%	–	>10 μM, 0% at 0.1nM to 10 μM	–	[80]
Mouse N18TG2 cell membranes	0.65 μM secretin	–	–	–	–	–	–	–	–	1000* 45%	–	–	–	[94]

(Table 4), contd.....

Preparation	Mode of cyclic AMP stimulation	Inhibitory EC_{50} values (nM) and maximal degree of inhibition (%)												
		CP559	CP552	CP505	THC	HU	Nab	(+)-WIN	(-)-WIN	AEA	2-AG	PEA	CBN	Ref.
Mouse N18TG2 cells	1 μM prostacyclin	–	–	– 50% at 30 μM	– 30% at 30 μM	–	–	–	–	–	–	–	–	[71]
Mouse N18TG2 cells	0.5 μM secretin	–	–	–	–	1.8 53%	–	–	–	–	–	–	–	[74]
Mouse N18TG2 cells	1 μM FSK	–	–	–	1.2 -35%	–	–	–	–	–	–	–	–	[85]
Mouse N18TG2 cells	1 μM FSK	–	–	–	35 45%	–	–	–	–	–	–	–	–	[34]
Mouse NG108-15 cells	1 μM FSK	–	–	–	–	–	16.2 35.6%	–	–	–	–	–	–	[87]
SH-SY5Y cells	1 μM FSK	–	–	–	–	–	20.2 45.4%	–	–	–	–	–	–	[87]
Rat cortical neurones (primary culture)	3 μM FSK	–	–	–	–	–	–	–	–	1200 >30%	800 >40%	–	–	[92]
Rat cortical neurones (primary culture)	1 μM FSK	4.6 54%	–	–	–	–	–	65 73%	–	–	–	–	–	[18]
Rat cortical neurones (primary culture)	1 μM isoprenaline	1 36%	–	–	–	–	–	5.1 59%	–	–	–	–	–	[18]
Rat cerebellar neurones (primary culture)	1 μM FSK	1.9	–	–	–	–	–	–	–	–	–	–	–	[18]
Rat cerebellar granule cells (primary culture)	1 μM FSK	220 65%	–	120 65%	2400 60%	–	–	410 48%	>10 μM <10% at 10 nM at 10 μM	–	–	–	–	[79]
Rat cerebellar membranes	None (basal)	60 40%	–	–	–	–	–	100 41%	–	1900 33%	–	–	–	[82]
Rat cerebellar membranes	None (basal)	–	–	200 40%	–	–	–	100 40%	–	–	–	–	–	[79]
Rat cerebellar membranes	FSK or basal	–	–	–	–	–	–	320 32%	>10 μM	–	–	–	–	[76]
Rat striatal membranes	FSK or basal	–	–	–	–	–	–	400 28%	–	–	–	–	–	[76]
Rat striatal neurones (primary culture)	1 μM FSK	3.5 ~40%	–	–	–	–	–	–	–	–	–	–	–	[18]
Rat cerebellar membranes	None (basal)	150 32%	–	–	–	–	–	210 35%	–	–	–	–	–	[83]
Rat striatal membranes	None (basal)	310 25%	–	–	–	–	–	200 29%	–	–	–	–	–	[83]
Rat substantia nigra membranes	FSK	–	–	–	–	–	–	40 35%	–	–	–	–	–	[30]
Mouse cerebellum membranes	1 μM FSK	72 35%	–	–	–	–	–	–	–	–	–	–	–	[90]

* In the presence of 10 to 200 μM phenylmethylsulphonyl fluoride.
# Effects of cannabinoids on secretin-stimulated cAMP production also investigated [70].
AEA, anandamide (Fig. 1); 2-AG, 2-arachidonoyl glycerol (Fig. 1); CBN, cannabinol (Fig. 8); CHO, Chinese hamster ovary; CP559, CP55940 (Fig. 3); CP552, CP55244 (Fig. 3); HU, HU-210 (Fig. 6); CP505, CP50556 (Fig. 3); Nab, nabilone (Fig. 2); PEA, palmitoylethanolamide (Fig. 1); THC, Δ^9-THC (Fig. 2); WIN, WIN55212 (Fig. 4).

Some cannabinoid receptor agonists have relatively low CB_1 and/or CB_2 efficacies. The pharmacological properties of such 'efficacy-driven' agonists is expected to show a particularly strong tissue-dependence. In a tissue preparation that contains a high concentration of cannabinoid receptors so that there is a significant 'receptor reserve', the size of the maximal response to an efficacy-driven agonist may be the same as that to a high-efficacy (affinity-driven) agonist, giving the false impression that both compounds have the same (high) efficacy. On the other hand, in a tissue preparation containing far fewer cannabinoid receptors (no 'receptor reserve'), the same efficacy-driven agonist may behave as a partial agonist or antagonist (e.g. see O-823 in next section). Hence if the relative efficacies of cannabinoid receptor agonists are determined by comparing maximal response size, it is important that this has been done using data obtained in the same tissue preparation and that the receptor population of this preparation (a) is small enough so that there is no significant receptor reserve, yet (b) is large enough to detect responses to low-efficacy compounds. Other reasons for using the same tissue preparation to determine the relative efficacies of cannabinoid receptor agonists are firstly, that there may be intertissue variations in the abilities of CB_1 or CB_2 receptors to amplify agonist-receptor interactions and secondly, that some tissues may

contain CB_1 or CB_2 receptor subtypes with their own distinct recognition sites or effector mechanisms. Already there is evidence that the efficiency with which G proteins transduce the agonist-cannabinoid receptor interaction can vary, at least within the brain. Thus there are several reports that the efficiency of coupling of cannabinoid receptors to G proteins, as defined for example by the ratio of apparent B_{max} of maximal cannabinoid receptor-stimulated [^{35}S]GTPγS binding to cannabinoid receptor B_{max}, is not the same in all brain areas [19, 22, 55, 69]. Indeed, there seem to be some brain areas, for example hypothalamus, that contain cannabinoid receptors with high coupling efficiency but in low concentration and other brain areas, for example hippocampus, that contain cannabinoid receptors with low coupling efficiency but in high concentration [22]. Consequently, the responsiveness of different brain areas to high-efficacy (affinity-driven) and low-efficacy (efficacy-driven) agonists may not always be highest in brain areas that contain the most cannabinoid receptors. Cannabinoid receptor coupling efficiency may also be affected by prior exposure to cannabinoids [19].

In the [^{35}S]GTPγS assay, increases in GDP concentration seem to magnify differences between the efficacies of different agonists such that the optimal GDP concentration appears to be higher for the assay of high-efficacy than low-efficacy agonists [67]. Indeed, elevating the concentration of GDP may completely abolish the ability of a low efficacy agonist to increase [^{35}S]GTPγS binding above basal levels. Compare for example the activities of the high-efficacy agonist, (+)-WIN55212 and the lower efficacy agonist Δ^9-THC observed in this assay by Griffin *et al.* [64]. Whereas (+)-WIN55212 produced greater stimulation of [^{35}S]GTPγS binding (over basal) at 100 μM GDP than at 10 μM GDP, Δ^9-THC stimulated [^{35}S]GTPγS binding in the presence of 10 μM GDP but not 100 μM GDP.

Cannabinoid Receptor Ligands

Classical Cannabinoids

The best known member of this group is Δ^8-tetrahydrocannabinol (Δ^9-THC) (Fig. 2). This is the main psychotropic constituent of cannabis and like all classical cannabinoids, it is a dibenzopyran derivative. Δ^9-THC undergoes significant binding to cannabinoid receptors at submicromolar concentrations, with similar affinities for CB_1 and CB_2 receptors (Table 2). At CB_1 receptors, Δ^9-THC behaves as an affinity-driven agonist, the sizes of its maximal effects in several receptor-containing tissue preparations falling below those of higher efficacy cannabinoid receptor agonists such as CP55940 and (+)-WIN55212 (Tables 3 and 4). As is to be expected for an affinity-driven agonist, the efficacy of Δ^9-THC shows 'tissue-dependence', there being some CB_1 bioassay systems in which it can elicit maximal responses that match those of higher efficacy agonists (Table 4). Δ^8-THC (Fig. 7), which has affinities for CB_1 and CB_2 receptors that are similar to those of Δ^9-THC (Table 2), also behaves as an affinity-driven agonist at CB_1 receptors [75, 77]. As to the CB_2 efficacy of Δ^9-THC, this is even less than at CB_1 receptors (Tables 4 and 5). Indeed, in some investigations Δ^9-THC has failed to show any agonist activity at all at CB_2 receptors and, in one set of experiments performed with CHO cells transfected with human CB_2 receptors and using the cyclic AMP assay, it behaved as a CB_2 receptor antagonist [34]. There are also some reports that Δ^9-THC behaves as an antagonist at the CB_1 receptor both in the [^{35}S]GTPγS

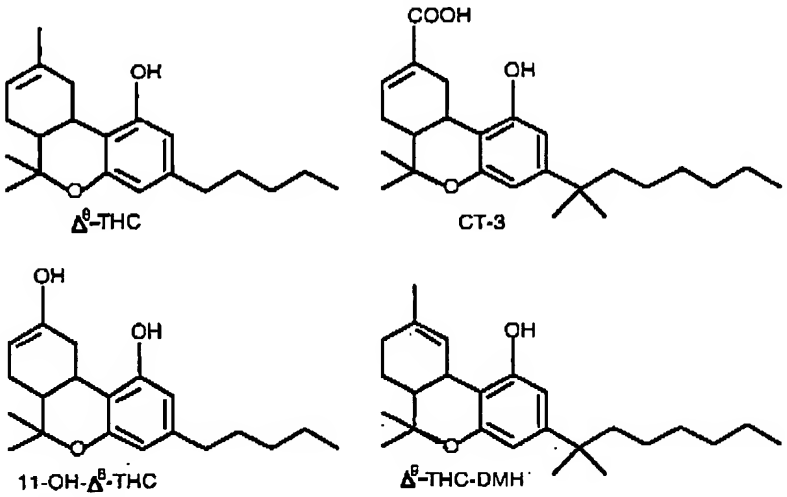


Fig. (7). Structures of four classical cannabinoids (DMH, dimethylheptyl).

assay performed with rat cerebellar membranes [19, 64] and when the measured response is (+)-WIN55212-induced inhibition of glutamatergic synaptic transmission in rat cultured hippocampal neurones [102].

Two other plant cannabinoids, cannabinol and cannabidiol (Fig. 8), have less affinity for cannabinoid receptors than Δ^8- or Δ^9-THC (Table 2). At CB_1 receptors, cannabinol behaves as an affinity-driven agonist with even less efficacy than Δ^9-THC (Tables 3 and 4). There is also one report that it behaves as an agonist for CB_2 receptors in the cyclic AMP assay [41]. However, in the GTPγS assay, cannabinol has been found to exhibit the properties of an inverse agonist for CB_2 receptors (Table 7). (The concept of inverse agonism is discussed later). Cannabidiol lacks significant agonist activity, at least at CB_1 receptors, as

Table 5. Inhibitory Effect of Certain Cannabinoids on Forskolin-stimulated Cyclic AMP Production by Cultured Cells Expressing Cannabinoid CB_2 Receptors

Preparation	Forskolin (μM)	Inhibitory EC_{50} values (nM) and maximal degree of inhibition (%)								
		CP559	THC	HU	(+)-WIN	(–)-WIN	AEA	2-AG	CBN	Ref.
Human CB_2-transfected CHO cells	?	2.51	–	–	–	–	–	–	–	[30]
Human CB_2-transfected CHO cells	5	0.72 ~55%	– 0%	0.37 ~50%	0.63 ~45%	>1 μM 0% at 1 μM or less	– 0%	–	–	[12]
Human CB_2-transfected CHO cells	?	2.89	41.8	0.578	0.407	–	957	–	>1 μM	[32]
Human CB_2-transfected CHO cells	1	–	>1 μM 0% at 0.01 to 1000 nM	1 ~48%	–	–	>1 μM* 0% at 0.01 to 1000 nM	–	–	[14]
Human CB_2-transfected COS cells	1	–	>1 μM <10% at 1 to 1000 nM	–	–	–	–	–	–	[34]
Human CB_2-transfected CHO cells	5	2 ~80%	–	–	3 ~75%	–	–	–	–	[94]
Human CB_2-transfected COS cells	1	–	>1 μM 21% at 1 μM	0.076	–	–	–	–	261	[41]
Human CB_2-transfected CHO cells	3	2.51 90%	–	–	–	–	–	–	–	[43]
Human CB_2-transfected 293 cells	0.5	52.1 60%	–	–	12.3 80%	–	–	–	–	[93]
Human CB_2-transfected CHO cells	3	– 76% at 100 nM	– 45% at 100 nM	–	– 80% at 100 nM	–	– 38% at 1 μM	–	–	[35]
Mouse CB_2-transfected CHO cells	3	– 75% at 100 nM	– 63% at 100 nM	–	– 62% at 100 nM	–	– 64% at 1 μM	–	–	[35]
CB_2-transfected COS cells	1	–	–	–	–	–	–	784* ~60%	–	[44]

* In the presence of 150 or 200 μM phenylmethylsulphonyl fluoride.
AEA, anandamide (Fig. 1); 2-AG, 2-arachidonoyl glycerol (Fig. 1); CBN, cannabinol (Fig. 8); CHO, Chinese hamster ovary; CP559, CP55940 (Fig. 3); HU, HU-210 (Fig. 6); THC, Δ^9-THC (Fig. 2); WIN, WIN55212 (Fig. 4).

Table 6. Inhibitory Effect of Cannabinoids on Voltage-activated Calcium Currents In Membranes of Cultured Cells Expressing Cannabinoid CB_1 Receptors

Preparation	Agonist	Agonist concentration (nM)	EC_{50} (nM)	Inhibition (%)	Ref
AtT-20 cells transfected with human CB_1 receptors	(+)-WIN55212	100	–	12% by 100 nM	[32]
AtT-20 cells transfected with rat CB1 receptors	(+)-WIN55212 Anandamide	1 to 300† 300	11 –	38% (Emax) 34% by 300 nM	[13]
Rat superior cervical ganglion cultured neurones transfected with rat CB_1 receptors	(+)-WIN55212 CP55940 Anandamide	1 to 3000† 1 to 10000 100	47 7 –	73% (Emax) 38% (Emax) 0 to 40% by 100 nM	[99]
NG108-15 neuroblastoma-glioma cells	Δ^9-THC CP55940	30 µM† 1 µM†	– –	40% 40%	[96]
NG108-15 neuroblastoma-glioma cells	(+)-WIN55212 CP55940	1 to 1000† 100	~3 –	43% (Emax) 36% by 100 nM	[95]
N18 neuroblastoma cells	(+)-WIN55212 Anandamide	100 1 to 1000†	– 20	54% by 100 nM 33% (Emax)	[97]
N18 neuroblastoma cells	(+)-WIN55212 Anandamide	~3 to 100 ~10 to 300	~10 ~10	~43% (Emax) ~30% (Emax)	[81]
N18 neuroblastoma cells	Mead ethanolamide	30 to 1000	124	~60% (Emax)	[98]
Rat hippocampal cultured neurones	(+)-WIN55212 (+)-WIN55212 CP55940 Anandamide	1 to 300 100*† 100 300	21 – – –	33% (Emax) 29.5% by 100 nM 25.5% by 100 nM 28.6% by 300 nM	[100]
Rat hippocampal	(+)-WIN55212	30 to 3000*	14	~23% (Emax)	[101]

cultured neurones should be in Table
*Antagonized by 200 or 300 nM SR141716A.
†Pertussis toxin sensitive.
Mead ethanolamide, 5Z, 8Z, 11Z-eicosatrienoic acid.

do cannabichromene and cannabigerol, also both plant cannabinoids. A high concentration (10 µM) of each of these three compounds failed to inhibit secretin-stimulated cyclic AMP production by N18TG2 cell membranes [72]. There is also a report that 10 µM cannabidiol does not enhance [^{35}S]GTPγS binding to rat cerebellar membranes [65]. Instead it was found to antagonize the stimulatory effect of CP55940 in this assay. Under the same conditions, 10 µM cannabinol did not antagonize CP55940 [65].

The affinities of Δ^9-THC, cannabinol, 11-hydroxy-Δ^8-THC and 11-hydroxy-cannabinol for cannabinoid receptors can be markedly enhanced by increasing the length of the alkyl side chain and by introducing methyl substituents into the lengthened side chain to produce dimethylheptyl analogues of these compounds (Table 2 and Figs. 6 to 8). These affinity increases are not accompanied by any marked changes in relative affinity for CB_1 and CB_2 receptors. The same changes in the alkyl side chain also enhance CB_1 and CB_2 receptor functional potency and efficacy. For example, in contrast to Δ^8-THC [75, 77], 11-OH-Δ^8-THC-dimethylheptyl (HU-210) has E_{max} values at CB_1 receptors that approximate to those of the high-efficacy cannabinoid receptor agonist, CP55940 (Tables 3 and 4). Unlike Δ^8-THC, HU-210 contains an 11-OH group. However, there is no evidence that insertion of this group produces any notable efficacy increase. Thus although 11-OH-Δ^8-THC is about 2 times more potent than Δ^8-THC as an inhibitor of secretin-stimulated cyclic AMP production in N18TG2 cell membranes, the maximal degree of inhibition produced by the two compounds is the same [72]. Similarly, in [^{35}S]GTPγS binding assays performed with rat cerebellar membranes, whilst 11-OH-Δ^9-THC is more potent than Δ^9-THC, the two compounds each elicit maximal stimulatory responses of about the same size [62]. The dimethylheptyl and 11-OH-dimethylheptyl analogues of cannabinol are also potent CB_1 and CB_2 receptor agonists. Their EC_{50} values for inhibition of forskolin-stimulated cyclic AMP production are 0.18 and 0.056 nM respectively in CHO cells stably transfected with rat CB_1 receptors and 0.79 and 0.208 nM respectively in CHO cells stably transfected with human CB_2 receptors [41]. The corresponding EC_{50} values for cannabinol are 120 and 261 nM respectively [41]. Whether these dimethylheptyl and 11-OH-dimethylheptyl analogues have greater efficacy than cannabinol remains to be announced. It has been reported, however, that 11-OH-cannabinol has negligible agonist activity at CB_2 receptors [41].

The degree of rigidity of the alkyl side chain also seems to have pharmacological importance, there being evidence that the introduction of a carbon-carbon triple bond into this part of the molecule reduces efficacy at CB_1 and CB_2 receptors without

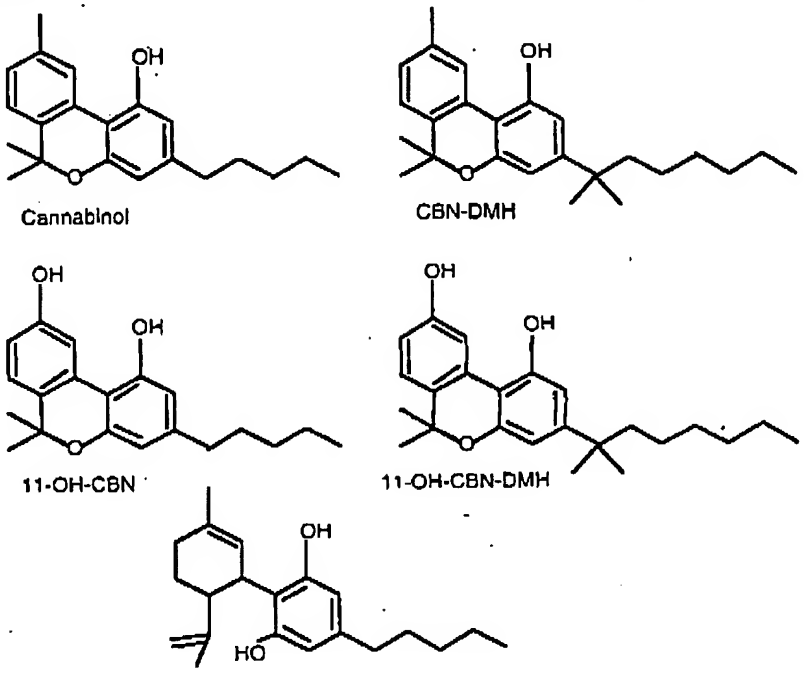


Fig. (8). Structures of cannabinol and cannabidiol and of three analogues of cannabinol (CBN, cannabinol; DMH, dimethylheptyl).

decreasing affinity for these receptor types [26, 36, 49, 103]. Thus 6'-azidohex-2'-yne-Δ^8-THC (O-1184; Fig. 9) has greater affinity than Δ^8-THC for both CB_1 and CB_2 receptors (Table 2). Yet, it behaves as a weak inverse agonist at CB_2 receptors [49] and, at CB_1 receptors, may have even lower efficacy than Δ^8-THC, behaving as a partial agonist in one assay system (inhibition of forskolin-stimulated cyclic AMP production by CHO cells transfected with human CB_1 receptors [49]) and as an antagonist in another [26]. A second classical cannabinoid with a 3-bond ynyl-containing side chain that seems to have high cannabinoid receptor affinity but reduced efficacy is 6'-cyanohex-2'-yne-Δ^8-THC (O-823; Fig. 9). The K_i value of this compound for displacement of [^{3}H]CP55940 from CB_1 binding sites is 0.77 nM. However, whilst it behaves as a potent cannabinoid receptor agonist in one CB_1-containing bioassay system (the mouse isolated vas deferens preparation), in another it behaves as a potent antagonist (myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine) [104]. Using tissue obtained from Δ^9-THC-tolerant mice, it became possible to demonstrate O-823-induced antagonism of a cannabinoid receptor agonist in the vas deferens as well, suggesting that like O-1184, O-823 is a potent affinity-driven cannabinoid receptor agonist and that it behaves as an agonist in tissues in which cannabinoid receptors are present in relatively high concentrations and/or have relatively efficient coupling to their second messenger system (the vas deferens) and as an antagonist in tissues in which these receptors are present at lower concentrations and/or are less efficiently coupled (myenteric plexus-longitudinal muscle preparation and cannabinoid tolerant vas deferens).

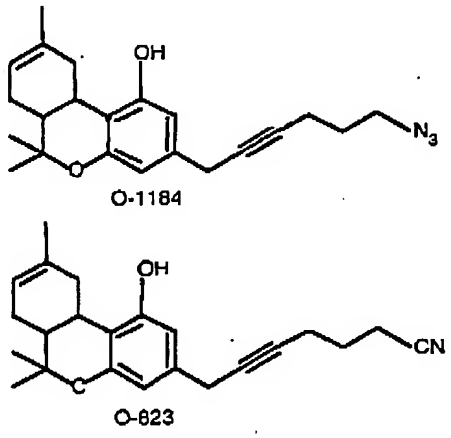


Fig. (9). Structures of O-1184 and O-823.

The results obtained with ynyl compounds such as O-1184 and O-823 provide clues as to how the structural features of classical cannabinoids that govern affinity for CB_1 and CB_2 receptors differ from those that determine efficacy at these receptors. So too do data obtained from experiments with the dimethylheptyl analogue of Δ^8-THC-11-oic acid (CT-3; Fig. 7) using CHO or COS cells transfected with rat CB_1 or human CB_2 receptors [41]. These experiments showed CT-3 to have about 8 times greater functional potency at the

Table 7. Inhibitory Effect of Certain Cannabinoid Receptor Ligands on GTPγS Binding by Tissue or Cultured Cell Membranes Expressing Cannabinoid CB_1 or CB_2 Receptors

Preparation	Experimental Conditions	EC_{50} values (nM) and maximal degree of inhibition relative to basal (%)				
		SR141	SR144	AM630	CBN	Ref.
Human CB_1-transfected CHO cell membranes	0.1 nM [^{35}S]GTPγS; 50 μM GDP; 100 mM NaCl; 3 mM $MgCl_2$; 30 μg protein; 0.2 mM EGTA; 0.1% BSA; 60 min at 30°C (0.2 ml assay)	– -25% by 100 nM	–	–	–	[59]
Human CB_1-transfected CHO cell membranes	0.1 nM [^{35}S]GTPγS; 50 μM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 0.1% tissue; 1 mM EDTA; 0.25% BSA; 90 min at 30°C (1 ml assay)	0.82* 22.3%	–	–	–	[60]
Human CB_1-transfected CHO cell membranes	0.1 nM [^{35}S]GTPγS; 50 μM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 0.1% tissue; 1 mM EDTA; 0.25% BSA; 90 min at 30°C (1 ml assay)	–	–	900* 20.9%	–	[63]
Human CB_1-transfected CHO cell membranes	1 nM [^{35}S]GTPγS; 320 μM GDP; 100 mM NaCl; 32 mM $MgCl_2$; 5 μg protein; 60 min at 37°C (0.25 ml assay)	5.5 47%	–	–	–	[66]
Rat cerebellum membranes	0.2 nM [^{35}S]GTPγS; 100 μM GDP; 1 mM $MgCl_2$; 15 μg protein; 1 mM EGTA; 0.25 mg BSA; 90 min at 30°C (0.5 ml assay)	>10 μM slight inhibition at 10 μM	–	–	–	[62]
Mouse whole brain membranes	0.1 nM [^{35}S]GTPγS; 50 μM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 1 mM EDTA; 90 min at 30°C (1 ml assay)	–	–	>0.1 mM* ~0%†	–	[56]
Guinea-pig whole brain membranes	0.1 nM [^{35}S]GTPγS; 50 μM GDP; 150 mM NaCl; 2.5 mM $MgCl_2$; 1 mM EDTA; 90 min at 30°C (1 ml assay)	–	–	>0.1 mM* ~0%†	–	[58]
Human CB_2-transfected CHO cell membranes	1 nM [^{35}S]GTPγS; 320 μM GDP; 100 mM NaCl; 32 mM $MgCl_2$; 5 μg protein; 60 min at 37°C (0.25 ml assay)	1000 65%	–	–	575.4 53%	[66]
Human CB_2-transfected CHO cell membranes	0.1 nM [^{35}S]GTPγS; 20 μM GDP; 100 mM NaCl; 10 mM $MgCl_2$; 20 μg protein; 0.2 mM EDTA; 90 min at 30°C (0.5 ml assay)	–	10.4 49%	76.6 47%	–	[47]

* In the presence of 100 μM phenylmethylsulphonyl fluoride.
† At 100 μM AM630.
BSA, bovine serum albumin; CBN, cannabinol (Fig. 8); CHO, Chinese hamster ovary; SR141, SR141716A (Fig. 5); SR144, SR144528 (Fig. 5). See Fig. 13 for the structure of AM630.

CB_2 than the CB_1 receptors, when the measured response is inhibition of forskolin-stimulated cyclic AMP production (EC_{50} = 116.2 and 927 nM respectively), but about 5 times greater binding potency at the CB_1 than the CB_2 receptors (Table 2).

Several CB_2-selective classical cannabinoids have now been developed (Fig. 10) [33, 38]. Huffman *et al.* [38] discovered that whilst removal of the phenolic OH group from HU-210 to form 1-deoxy-11-OH-Δ^8-THC-dimethylheptyl (JWH-051) did not significantly affect affinity for CB_1 receptors, it greatly enhanced CB_2 receptor affinity (Table 2). As a result, JWH-051 was found to have 37.5 fold selectivity for CB_2 receptors. Even more dramatic is the CB_2-selectivity shown in binding experiments by JWH-133 and JWH-139 and by the Merk-Frosst compounds, L759633 and L759656 (Table 2). The CB_2 receptor affinity of JWH-139, L759633 and L759656 is somewhat less than that of JWH-051 or JWH-133, although still in the low nM

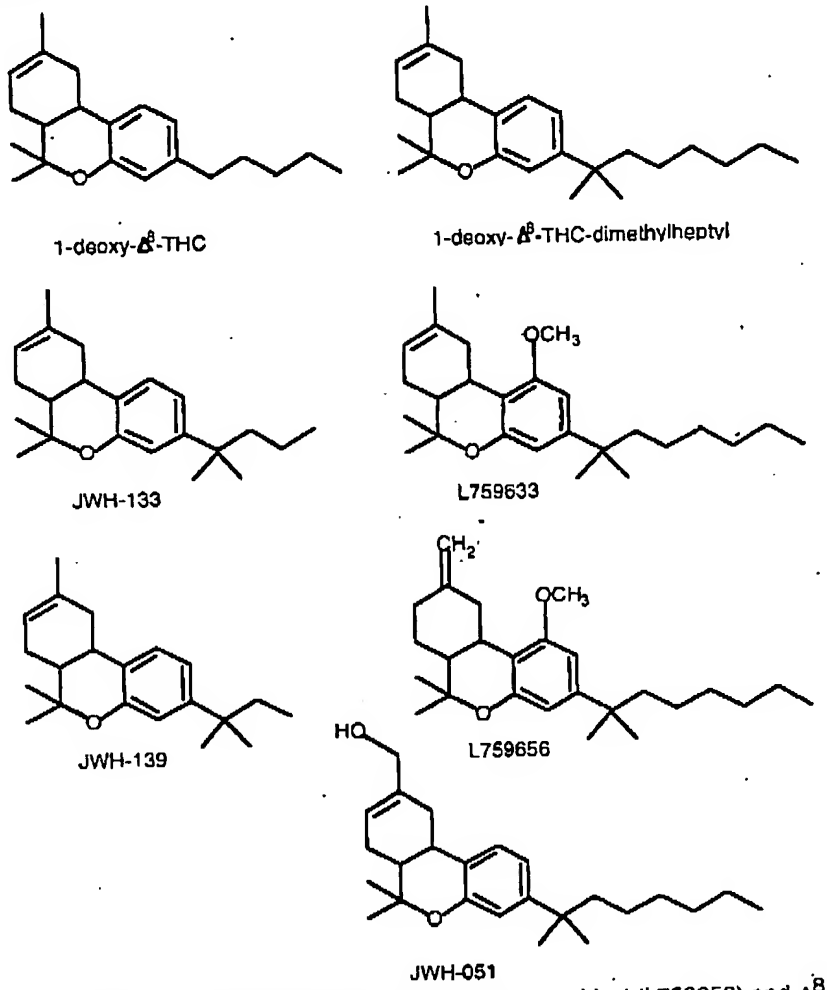


Fig. (10). Structures of some deoxy or methoxy analogues of hexahydrocannabinol (L759656) and Δ^8-THC.

range. More important; however, is the very much lower affinity JWH-133, JWH-139 and the two Merk-Frosst compounds have for CB_1 receptors than JWH-051. L759633 and L759656 seem to be CB_2 receptor agonists with both high efficacy and high potency. Thus Ross *et al.* [47] have reported firstly, that each of these compounds is approximately equipotent with the high-efficacy cannabinoid receptor agonist, CP55940, in inhibiting forskolin-stimulated cyclic AMP production by human CB_2 receptor transfected CHO cells and secondly, that the maximum degree of inhibition produced in this assay by all three compounds is the same. They found L759656 (10 μM) to be inactive at CB_1 receptors and L759633 to behave as a weak CB_1 receptor agonist with an EC_{50} of about 10 μM. Although there is good evidence from in vivo experiments that JWH-051 is a potent CB_1 receptor agonist [38] there are no reports as to whether it is also a CB_2 receptor agonist. Whether it will prove possible to develop agonists from classical cannabinoids that exhibit the same high degree of selectivity for CB_1 receptors that the Merk-Frosst compounds show for CB_2 receptors remains to be established.

Finally, for Δ^8-THC, Δ^9-THC and other classical cannabinoids with chiral centres, it is the (–)-enantiomers that show the greater CB_1 and CB_2 receptor affinity and functional potency [1]. This stereoselectivity is well illustrated by comparing the CB_1 or CB_2 affinities of (–)- and (+)-11-OH-dimethylheptyl-Δ^8-THC (HU-210 and HU211 respectively) (Table 2) or their potencies in CB_1 and CB_2 receptor-containing bioassay systems [12, 74, 105].

Non-classical Cannabinoids

This group of cannabinoids was developed by a Pfizer research team [106]. It consists of bicyclic and tricyclic analogues of Δ^9-THC that lack a pyran ring. A particularly important member of this group of compounds is the cannabinoid receptor agonist, CP55940 (Fig. 3) as it is the tritiated form of this compound that was used to demonstrate for the first time that brain tissue contains specific cannabinoid binding sites [107]. It was this landmark observation in particular that led to the realization that cannabinoids

act through receptors. CP55940 binds equally well to CB_1 and CB_2 receptors (Table 2). It shows high affinity for both receptor types, explaining the usefulness of radiolabelled CP55940 as a probe for CB_1 and CB_2 receptors. CP55940 behaves as an efficacy-driven agonist for both receptor types, the sizes of the effects it produces at maximal concentrations in CB_1 and CB_2 receptor assay systems often matching or exceeding the maximal responses to several other cannabinoid receptor agonists (Tables 3 to 5). Evidence that CP55940 does not need to occupy all cannabinoid receptors in the cerebellum to elicit a maximal response lends further support to its classification as a high-efficacy agonist [90]. Other non-classical cannabinoids often used as experimental tools are CP55244 and CP50556 (L-nantradol) (Fig. 3), and desacetyl-L-nantradol [1]. These also behave as high-affinity, high-efficacy agonists, at least for CB_1 receptors (Tables 3 and 4 and [1]). Indeed, CP50556 and CP55244 may have even higher CB_1 efficacy than CP55940 (Table 3 and [67]). It seems likely that these other non-classical cannabinoids share the ability of CP55940 to interact with CB_2 receptors. However, this remains to be established. Like classical cannabinoids, non-classical cannabinoids with chiral centres exhibit significant stereoselectivity, the (−)-enantiomers having the greater activity (Table 2 and [1]).

Eicosanoids

The prototypic member of the eicosanoid group of cannabinoid receptor agonists is arachidonoylethanolamide (anandamide) (Fig. 1). This is the first of four endogenous cannabinoid receptor agonists to have been discovered in mammalian brain and other tissues, the others being 2-arachidonoyl glycerol, homo-γ-linolenoylethanolamide and docosatetraenoylethanolamide [1, 2, 5, 108]. Of these, the most investigated and probably also the most important physiologically, are anandamide and 2-arachidonoyl glycerol.

Anandamide and Related Fatty Acid Amides

Anandamide binds significantly to both types of cannabinoid receptor with marginally higher affinity for the CB_1 receptor (Table 2). Indeed, when protected from hydrolysis (see below), its affinity for CB_1 receptors matches that of Δ^9-THC. Anandamide also resembles Δ^9-THC in other ways. Firstly, the affinity of anandamide for CB_1 receptors increases when its non-carboxylic hydrocarbon tail is lengthened and branched. Most notably, as for the alkyl side chain of Δ^9-THC, extension of the non-carboxylic tail of anandamide by two carbon atoms together with the introduction of two methyl substituents, produces an increase in both CB_1 binding affinity and in vivo potency for CB_1 receptor-mediated effects [109, 110]. Secondly, anandamide behaves as an affinity-driven CB_1 receptor agonist. Thus its efficacy at CB_1 receptors, although higher than that of Δ^9-THC, is often found to be lower than that of (+)-WIN55212 or CP55940 (Tables 3, 4 and 6). Thirdly, anandamide has much lower efficacy at CB_2 than CB_1 receptors, lacking detectable CB_2 activity altogether in some experiments (Table 5). These similarities between Δ^9-THC and anandamide are in line with results obtained in molecular modelling studies. Thus Thomas *et al.* [111] have reported that it is possible to superimpose anandamide on the Δ^9-THC molecule such that the oxygen of the arachidonoyl carboxyamide lies over the pyran oxygen, the hydroxyl group of the arachidonoyl ethanol over the phenolic hydroxyl group, the five terminal arachidonoyl carbons over the hydrophobic pentyl side chain and the arachidonoyl polyolefin loop over the tricyclic ring system. A fuller account of the structure-activity relationships in the anandamide series is to be found in two recent papers [45, 108]. It should be noted that the maximal effects of anandamide are not always less than those of WIN55212 or CP55940 (Tables 4 and 6). For example, in AtT-20 cells transfected with rat CB_1 receptors, the inhibition of an inwardly rectifying potassium current by 300 nM anandamide has been found to match the maximal inhibitory effect of WIN55212 [13].

Because anandamide is susceptible to hydrolysis by fatty acid amide hydrolase, in vitro assays of this agent are often carried out in the presence of an enzyme inhibitor such as phenylmethylsulphonyl fluoride [1, 2]. The extent to which anandamide has to be protected from enzymic hydrolysis in this way can be affected by the assay conditions used [1]. In binding assays, for example, there is evidence that whilst there may be a need to protect anandamide from enzymic hydrolysis when a filtration method is used to separate tissue samples from unbound radioligand, this precaution is not needed when the separation is achieved by centrifugation (all [^{3}H]HU-243 experiments in Table 2) [112]. In some cannabinoid receptor-containing tissue preparations, the potency of anandamide is not enhanced by phenylmethylsulphonyl fluoride. This may be because these preparations lack significant fatty acid amide hydrolase activity. However, it could also be because they are unable to take up anandamide from its presumed extracellular site of action, there being evidence that fatty acid amide hydrolase is located intracellularly so that released anandamide is only metabolized after it has been taken up into tissues both by a carrier-mediated process and by passive diffusion [5, 108].

The finding that anandamide is the substrate of an endogenous amide hydrolase has stimulated the development of several analogues that are less susceptible to enzymic hydrolysis. One of these is (R)-

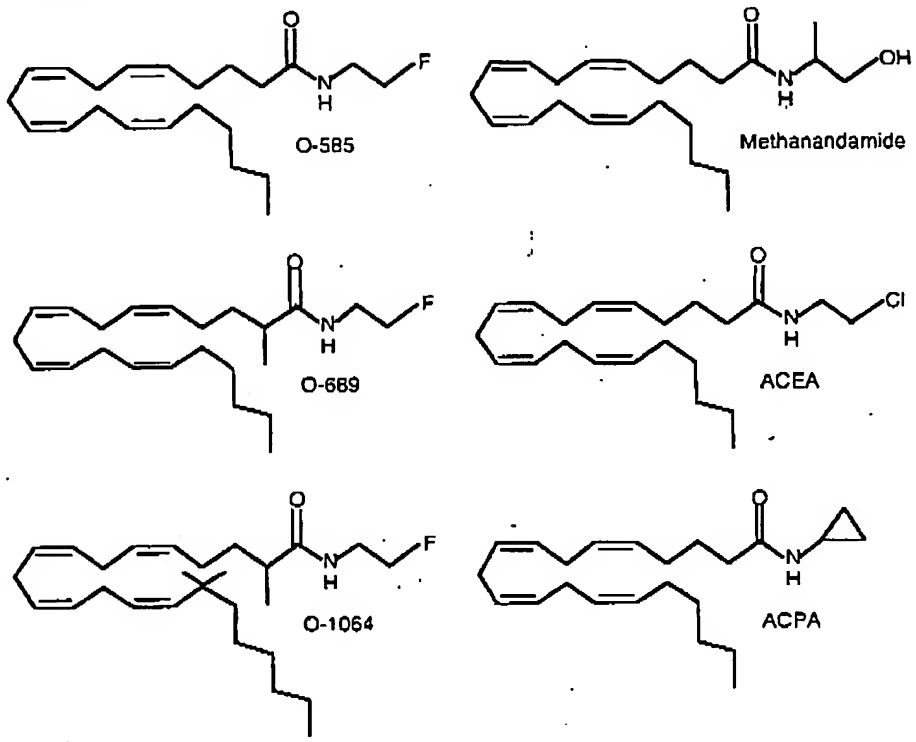


Fig. (11). Structures of some synthetic eicosanoid cannabinoids.

(+)-arachidonoyl-1'-hydroxy-2'propylamide (methanandamide; Fig. 11) [113]. Unlike anandamide, methanandamide possesses a chiral centre, its (R)-isomer possessing 9-fold higher CB_1 receptor affinity than its (S)-isomer [113]. Another analogue that shows resistance to enzymic hydrolysis is 2-methylarachidonoyl-(2'-fluoroethyl)amide (O-689; Fig. 11) [114] and it is noteworthy that both O-689 and methanandamide have an enhanced selectivity and affinity for CB_1 receptors (Table 2). Two anandamide analogues with even greater selectivity (and affinity) for CB_1 receptors are arachidonoyl-2'-chloroethylamide (ACEA) and arachidonoylcyclopropylamide (ACPA) (Table 2 and Fig. 11). The E_{max} values of both analogues, determined in the cyclic AMP assay using CHO cells transfected with human CB_1 receptors and in the GTPγS assay using rat cerebellar membranes, match those of the high-efficacy agonist, CP55940 [46]. However, ACPA (but not ACEA) was found to have a significantly lower E_{max} than CP55940 for inhibition of electrically-evoked contractions of the mouse isolated vas deferens. Neither ACEA nor ACPA showed any sign of reduced susceptibility to enzymic hydrolysis [46].

Two other anandamide analogues of note are N-(4-hydroxyphenyl) arachidonylamide (AM404) and methyl arachidonyl fluorophosphonate (MAFP) (Fig. 12) [40, 115, 116]. AM404 inhibits anandamide uptake by rat cultured cortical neurones (EC_{50} = 1 μM) and astrocytes (EC_{50} =5 μM) without also producing detectable inhibition of FAAH or inhibiting the uptake of the anandamide metabolites, arachidonic acid and ethanolamine [116]. In line with its properties, AM404 has been shown to potentiate some effects of anandamide, for example anandamide-induced inhibition of forskolin-stimulated cyclic AMP production by rat cultured cortical neurones, anandamide-induced antinociception in the mouse hot plate test and anandamide-induced hypotension in anaesthetized guinea-pigs [116, 117]. AM404 also binds to CB_1 receptors at micromolar concentrations (Table 2). However it does not behave as a direct cannabinoid receptor agonist [116] and its ability to potentiate anandamide suggests that it does not exert any significant degree of cannabinoid receptor antagonism either. MAFP is an irreversible ligand for the CB_1 receptor (EC_{50} = 20 nM for displacement of [^{3}H]CP55940 from specific binding sites on rat brain membranes) and also an irreversible inhibitor of FAAH (EC_{50} = 2.5 nM) [115]. As expected from its binding properties, MAFP (1 μM) has also been shown to produce insurmountable antagonism of several cannabinoid receptor agonists in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine, a functional CB_1 receptor assay system [118]. Other fatty acid derivatives that have proved to be potent irreversible inhibitors of FAAH are a series of saturated fatty acid sulphonyl fluorides [119, 120]. The most notable of these is stearyl sulphonyl fluoride

(AM381) as this shows the greatest separation between potency for FAAH inhibition (EC_{50} = 4 nM) and ability to bind to CB_1 receptors (EC_{50} = 18.5 μM for displacement of [^{3}H]CP55940 from specific binding sites on rat forebrain membranes). This is far greater than the 8-fold separation between FAAH inhibitory potency and CB_1 binding potency shown by MAFP. When taken together, the results obtained with stearyl sulphonyl fluoride and AM404 suggest that the structural prerequisites for inhibiting FAAH are not the same as those for inhibiting anandamide uptake or for binding to cannabinoid receptors. There is also evidence that the structural features that determine the ability to inhibit the anandamide transporter differ from those that determine the ability to serve as a substrate for the anandamide transporter [6, 121].

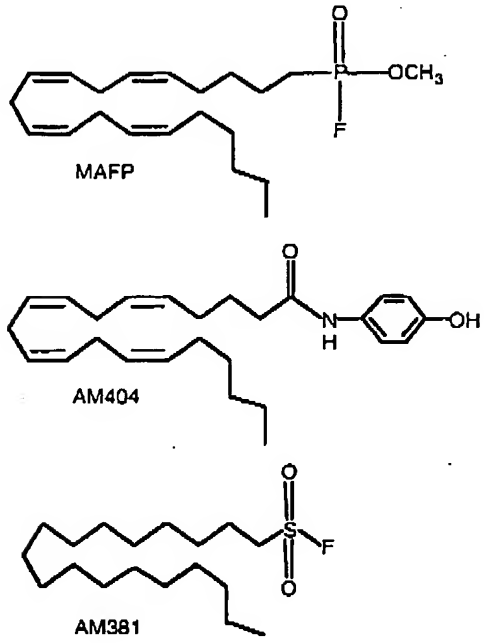


Fig. (12). Structures of methyl arachidonyl fluorophosphonate (MAFP), N-(4-hydroxyphenyl) arachidonylamide (AM404) and stearyl sulphonyl fluoride (AM381).

One endogenous fatty acid amide that has been the source of some confusion in the cannabinoid literature is palmitoylethanolamide (Fig. 1). This has been found by Facci *et al.* [122] to share the abilities of nabilone and (+)-WIN55212 to prevent antigen-induced degranulation of rat basophilic leukemia-2H3 cells (EC_{50} = 0.27 μM, 2.8 μM and 13 μM respectively). Like nabilone and anandamide (EC_{50} = 2.6 nM and 33 nM respectively), palmitoylethanolamide also displaced [^{3}H](+)-WIN-55212 from specific binding sites on membranes from the same cell line (EC_{50} = 1 nM). These effects of palmitoylethanolamide could not have been mediated by CB_1 receptors as the RBL-2H3 cells were found not to express CB_1 receptors [122] and as palmitoylethanolamide does not have significant affinity for CB_1 receptors in rat brain membranes or in membranes from CB_1 receptor transfected CQS cells [112, 123] or show any activity (at 10 μM) as an inhibitor of forskolin-stimulated cyclic AMP production in N18TG2 or rat CB_1 transfected CHO cells [80]. Although the RBL-2H3 cells were found to express CB_2 receptors [122], it is also unlikely that palmitoylethanolamide acted through these receptors. Thus Showalter *et al.* [37] have found that at concentrations of up to 10 μM, palmitoylethanolamide displaces only 20% of [^{3}H](+)-WIN-55212 from binding sites on membranes obtained from human CB_2 receptor transfected CHO cells and that this displacement is not concentration-dependent. Palmitoylethanolamide was found to be even less effective in displacing [^{3}H]CP55940 from these CB_2 binding sites [37]. This finding has been confirmed by Sheskin *et al.* [112] in competitive binding assays using membranes from CB_2 receptor transfected COS cells and [^{3}H]HU-243 as the radiolabelled probe. One possibility is that palmitoylethanolamide is a ligand for a novel type of cannabinoid receptor. Indeed, the suggestion has already been made that palmitoylethanolamide binds to a yet unidentified CB_2 receptor subtype [112]. This hypothesis has been used by Calignano *et al.* [124] as a possible explanation for their finding that the ability of palmitoylethanolamide, injected into the hind paws of mice, to prevent behavioural signs of hyperalgesia induced by intraplantar injection of dilute formalin can be attenuated by the CB_2 receptor antagonist, SR144528, but not by the CB_1 receptor antagonist, SR141716A. Evidence for the existence of a novel CB_2 receptor subtype has also been obtained in experiments with the mouse vas deferens [125].

2-Arachidonoyl Glycerol

Relatively little is yet known about the pharmacological properties of 2-arachidonoyl glycerol (Fig. 1). What data there are, suggest that this endogenous compound resembles anandamide in binding almost as well to CB_2 as to CB_1 receptors and that its affinities for both receptor types are similar to those of anandamide (Table 2). 2-Arachidonoyl glycerol has also been shown to behave as an agonist at both cannabinoid receptor types, inhibiting forskolin-stimulated cyclic AMP production in rat cultured cortical neurones with a potency slightly greater than that of anandamide [92] and inducing inhibition of forskolin-stimulated cyclic AMP production in COS cells transfected with CB_1 or CB_2 receptors [44], inhibition of depolarization-induced increases in free calcium within differentiated neuroblastoma x glioma hybrid NG108-15 cells [126] and inhibition of long-term potentiation in rat hippocampal slices [92]. The maximal degree of inhibition produced by 2-arachidonoyl glycerol in the

COS cell experiments has been reported to be less at CB_2 receptors (ca. 60%) than at CB_1 receptors (ca. 80%) [44], suggesting that like anandamide it may have less CB_2 than CB_1 efficacy. However, this remains to be confirmed.

Few structure-activity studies have been performed with arachidonoyl glycerol analogues, the available data suggesting that 1(3)-arachidonoyl glycerol (Fig. 1) has similar CB_1 and CB_2 binding properties to 2-arachidonoyl glycerol [108] and about 3 times greater potency than 2-arachidonoyl glycerol as an inhibitor of forskolin-stimulated cyclic AMP production in rat cultured cortical neurones [92], that 2-palmitoyl glycerol and 2-linoleoyl glycerol lack significant affinity for CB_1 or CB_2 receptors [31, 44, 108] and that 1(3)-palmitoyl glycerol and 1(3)-stearoyl glycerol (10 μM) do not share the ability of 1(3)- and 2-arachidonoyl glycerol to inhibit forskolin-stimulated cyclic AMP production in rat cultured cortical neurones [92].

The degree of inhibition of forskolin-stimulated cyclic AMP production induced by 10 μM 1(3)- or 2-arachidonoyl glycerol in rat cultured cortical neurones is almost as great as that induced by 3 μM (+)-WIN55212 [92]. Additional experiments are now required to establish whether this is an indication that 1(3)- and 2-arachidonoyl glycerol resemble (+)-WIN55212 in being high-efficacy CB_1 receptor agonists. It is noteworthy, however, that in vivo experiments with mice have already shown that the E_{max} of 2-arachidonoyl glycerol is similar to that of Δ^9-THC or anandamide for the production of hypokinesia and antinociception but less than the E_{max} values of these other cannabinoids for the production of hypothermia and catalepsy [31]. All four of these effects are thought to be CB_1-receptor mediated.

Aminoalkylindoles

This group of cannabinoid receptor ligands was developed by a Sterling Winthrop research team [76, 127] and contains compounds that are structurally quite different from classical, non-classical or eicosanoid cannabinoids. The prototype of this group is the cannabinoid receptor agonist, (+)-WIN55212 (Fig. 4). This binds with high affinity to both cannabinoid receptor types, exhibiting marginal selectivity for CB_2 receptors (Table 2) and behaves as a high-efficacy agonist at both CB_1 and CB_2 receptors ([1] and Tables 3 and 4 to 6). In contrast, (–)-WIN55212 does not interact significantly with either cannabinoid receptor type ([1] and Tables 3 to 5).

There is evidence that aminoalkylindoles bind differently to the CB_1 receptor than classical, non-classical or eicosanoid cannabinoids, albeit at a site that overlaps sufficiently with the binding site(s) of these other types of cannabinoid to allow mutual displacement between aminoalkylindole and non-aminoalkylindole cannabinoids to occur [1]. More specifically, Song and Bonner [16] obtained evidence that lysine^{192} is a critical point of interaction on the CB_1 receptor for HU-210, CP55940 and anandamide (see below) but not for (+)-WIN55212. They found that HU-210, CP55940 and anandamide had little inhibitory effect on cyclic AMP production by human embryonal kidney 293 cells transfected with CB_1 receptors in which lysine at position 192 had been replaced with alanine. The same mutation also abolished the ability these cannabinoids to compete with [^{3}H](+)-WIN55212 for specific binding sites but had little effect on the binding affinity of (+)-WIN55212 or on its ability to inhibit adenylate cyclase. More recently, Chin *et al.* [24] found that when mutant CB_1 receptors in which lysine^{192} is replaced with arginine were transfected into CHO cells, they retained the ability to bind and to respond functionally to both CP55940 and (+)-WIN55212. On the other hand, when lysine^{192} was replaced with glycine or glutamate, binding affinity was retained for (+)-WIN55212 but lost for CP55940. Tao and Abood [93] found that human embryonal kidney 293 cells expressing mutant CB_1 receptors in which aspartate^{163} had been replaced with asparagine or glutamate had less affinity for (+)-WIN55212 than cells expressing wild-type CB_1 receptors whereas these three cell lines did not differ significantly in their affinities for Δ^9-THC, CP55940, anandamide or SR141716A (see below). In the same investigation, binding of (+)-WIN55212, CP55940, Δ^9-THC and HU-210 to human embryonal kidney 293 cells expressing CB_2 receptors was found not to be affected by the replacement of aspartate^{80} with asparagine or glutamate. Other evidence that (+)-WIN55212 binds differently to CB_1 receptors than other types of ligand for this receptor is detailed elsewhere [1].

In CB_1 binding and functional assays, (+)-WIN55212 is often found to be less potent than CP55940 (Tables 2 to 4 and 6). However, as indicated by the data shown in Tables 3, 4 and 6, E_{max} values of (+)-WIN55212 determined in CB_1 receptor assay systems frequently exceed those of CP55940, raising the possibility that (+)-WIN55212 may have greater CB_1 receptor efficacy than CP55940. This possibility receives further support from the observations firstly, that the maximal inhibitory effect of (+)-WIN55212 (86%) on intracellular calcium-mediated 'spiking' activity of rat cultured hippocampal neurones caused by lowering the extracellular concentration of Mg^{++} is greater than that of CP55940 (44%) and secondly, that this inhibitory effect of (+)-WIN55212 can be attenuated by CP55940 when this is applied at a concentration of 100 nM [128]. In some CB_1 receptor assay systems, E_{max} values of CP55940 exceed those of (+)-WIN55212 (Tables 3 and 4). Variations in the rank order of the apparent efficacies of

(+)-WIN55212 and CP55940 could stem from differences in the manner in which these two agonists seem to interact with CB_1 receptors (see above), from inter-tissue differences in cannabinoid receptor density or from the presence of a cannabinoid receptor type that has yet to be detected [29, 100]. Results from the rather small number of experiments that have so far been carried out with CB_2 receptor assay systems usually show (+)-WIN55212 to have the same or greater potency than CP55940 but slightly lower efficacy (Tables 2, 3 and 5).

Several analogues of WIN55212 have been developed with significantly greater affinity for CB_2 than CB_1 receptors [37, 39, 47]. These include L768242, JWH-015 and 6-iodopravadoline (AM630), (Fig. 13 and Table 2). For L768242 and some related compounds [39] only binding data are available and so it is not known whether these compounds have CB_1 or CB_2 receptor efficacy. For JWH-015, there is some evidence from in vivo experiments that it is a CB_1 receptor agonist [129, 130]. Whether it is also a CB_2 receptor agonist remains to be established. More is known about the interactions of AM630 with CB_1 and CB_2 receptors. On the CB_1 side, this compound has been shown to inhibit forskolin-stimulated cyclic AMP production by human CB_1-transfected CHO cells with an EC_{50} well above 1 μM [47] and to cause a surmountable antagonism of (+)-WIN55212-induced stimulation of [^{35}S]GTPγS binding to mouse and guinea-pig brain membranes with K_B values of 3.1 and 9.3 μM respectively [56, 58]. These mixed agonist-antagonist properties of AM630 together with its low potency suggest this agent to be a low-affinity, low-efficacy agonist at CB_1 receptors. This conclusion is supported by findings that AM630 behaves as a weak CB_1 receptor agonist in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine (EC_{50} = 1.9 μM) [131] and that at a concentration of 100 μM it produces only a 7.5% increase in [^{35}S]GTPγS binding to guinea-pig brain membranes and has no appreciable stimulatory effect on [^{35}S]GTPγS binding to mouse brain membranes [56, 58]. There is one set of data that does not support the conclusion that AM630 is a CB_1 receptor partial agonist. This was obtained by Landsman *et al.* [63] who found AM630 to behave as an inverse agonist at CB_1 receptors. They demonstrated that AM630 can inhibit [^{35}S]GTPγS binding to membranes from human CB_1-transfected CHO cells by up to 20.9% (EC_{50} = 0.9 μM). It would seem, therefore, that depending on the CB_1 receptor preparation or assay used, AM630 can behave as an agonist, an antagonist or an inverse agonist.

At CB_2 receptors, AM630 seems to lack agonist activity, behaving instead as an antagonist or inverse agonist. In particular, experiments with human CB_2-

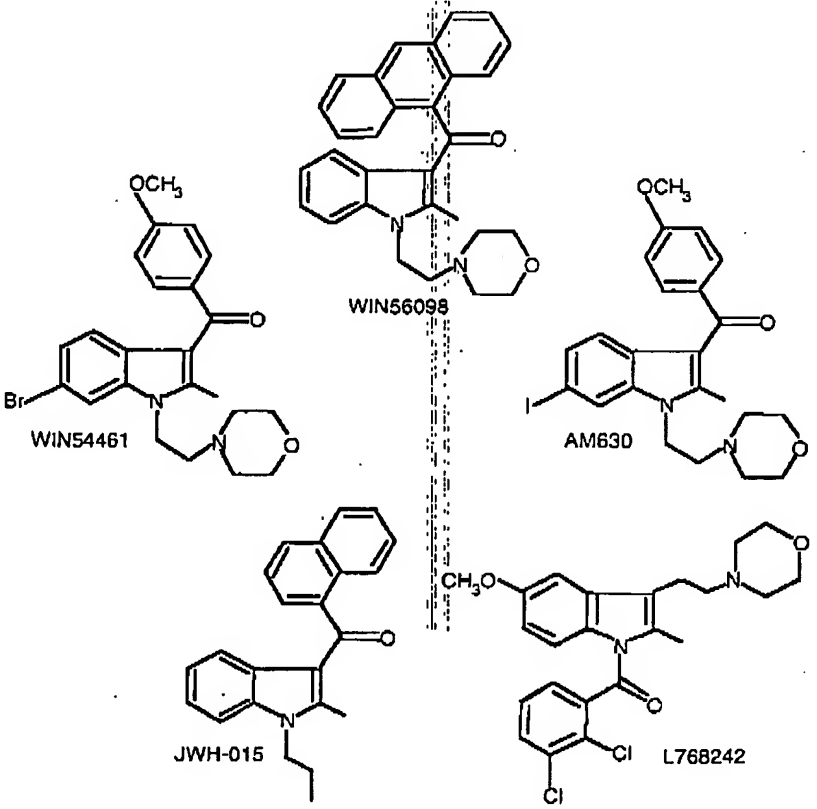


Fig. (13). Structures of some aminoalkylindole cannabinoids.

transfected CHO cell preparations have shown it to reverse CP55940-induced inhibition of forskolin-stimulated cyclic AMP production (EC_{50} = 129 nM), to enhance forskolin-stimulated cyclic AMP production (EC_{50} = 230 nM) and to inhibit [^{35}S]GTPγS binding (Table 7). These data may explain the ability of AM630 to antagonize inhibition of electrically-evoked contractions of the mouse isolated vas deferens induced by (+)-WIN55212, CP55940 and Δ^9-THC [132] as this tissue is now known to contain CB_2-like cannabinoid receptors [125]. Indeed the K_B values of AM630 against these compounds, 36.5, 17.3 and 14 nM, respectively, are of the same order as the K_i value of AM630 for displacement of [^{3}H]CP55940 from CB_2 binding sites (31.2 nM; Table 2). Two other WIN55212 analogues have been reported to produce surmountable attenuation of cannabinoid-induced inhibition of electrically-evoked contractions of the mouse isolated vas deferens. These are WIN56098 and 6-bromopravadoline (WIN54461) (Fig. 13). WIN56098 is less potent than AM630 with a K_B of 1.85 μM for antagonism of Δ^9-THC [76]. WIN54461 shows greater potency than WIN56098 as an antagonist in this assay system, with K_B values against (+)-WIN55212 and Δ^9-THC of 50 and 316 nM respectively [133, 134]. Its IC_{50} for displacement of [^{3}H]WIN55212 from rat cerebellar membranes has been reported to be 515 nM [134]. WIN54461 has been found by Griffin and Pertwee (unpublished) to behave as a partial agonist in the mouse vas deferens and does not show antagonist properties in vivo [134]. The in vivo pharmacology of WIN56098 and AM630 remains to be explored.

Diarylpyrazoles

SR141716A

SR141716A (Fig. 5) is a member of a series of more than one hundred and ninety four 1,5-diphenylpyrazoles developed by Sanofi Recherche during a search for a CB_1-selective antagonist [30, 50]. It binds with significantly higher affinity to CB_1 than CB_2 receptors (Table 2), lacks detectable cannabimimetic activity at CB_1 or CB_2 receptors and shows no significant affinity for a wide range of non-cannabinoid receptors [30, 35, 91]. There are also reports that [^{3}H]SR141716A is not displaced from its binding sites by ligands for a wide selection of non-cannabinoid receptors [15, 20]. SR141716A has been shown to prevent the production of many in vivo and in vitro effects that are thought to be mediated by CB_1 receptors, usually with a potency that is consistent with its high affinity for CB_1 binding sites. Examples include the ability of SR141716A to attenuate or reverse cannabinoid-induced inhibition of forskolin-stimulated cyclic AMP production in CB_1 transfected cells; cannabinoid-induced inhibition of electrically-evoked contractions of the mouse isolated vas deferens and cannabinoid-induced production of hypokinesia, antinociception, hypothermia and catalepsy in mice [1, 2]. In interpreting data obtained from experiments with SR141716A, it is important to bear in mind that although this compound is CB_1-selective it is not CB_1-specific. Thus whilst it is safe to assume that in tissues containing both CB_1 and CB_2 receptors, concentrations of SR141716 in the low or mid nanomolar range will interact mainly with CB_1 receptors; this is not so for higher concentrations of SR141716A.

Thomas *et al.* [25] have investigated the structure-activity relationships of SR141716A by performing displacement binding studies with analogues of SR141716A in which a hydrogen atom at carbon 3 or 6 of the dichlorophenyl group has been replaced with I or Cl and/or the 4'-Cl on the other phenyl group has been replaced with I, Br or H. Their results suggest that SR141716A can be superimposed on the Δ^9-THC molecule such that the 4' position of SR141716A lies over the pentyl side chain of Δ^9-THC, the lone pair electrons of the carbonyl oxygen of SR141716A over the pyran oxygen of Δ^9-THC and the lone pair electrons associated with the pyrazole pyridine nitrogen of SR141716A over the phenolic hydroxyl of Δ^9-THC. They also conclude that the dichlorinated ring system of SR141716A may be its 'antagonist conferring' region.

There is an ever growing number of reports that when administered by itself, often (but not always) at doses likely to be in the CB_1-selective range, SR141716A can produce in vivo or in vitro effects that are opposite in direction from those produced by cannabinoid receptor agonists. 'Inverse cannabimimetic effects' of SR141716A that have been observed in vivo include (a) depression of spontaneous firing of neurones in the ventral tegmental area (dopaminergic) or substantia nigra pars reticulata of anaesthetized rats [135, 136], (b) enhancement of spontaneous cortical and hippocampal acetylcholine release in unanaesthetized rats [137, 138], (c) enhancement of short-term working memory in adult or aged rats and mice [139] and of intestinal motility in mice [140, 141], (d) production of signs of anxiety in rats [142], of arousal in the rat sleep-waking cycle [143], of hyperalgesia in rats and mice [124, 144-146], of positive reinforcement in rats in a conditioned place preference test [147] and of hyperkinesia in mice [148] and (e) suppression of appetite for sucrose or standard diet in marmosets or rats [149-153] and of the establishment of food-, cocaine- or morphine-induced conditioned place preference in rats [151]. SR141716A has also been reported to elevate blood pressure in a rat model of haemorrhagic shock [154] and to suppress alcohol consumption, both by Sardinian alcohol-preferring rats

[153] and by C57BL/6 mice which also have a genetic predisposition for alcohol [152].

In vitro inverse cannabimimetic effects of SR141716A, all observed in experiments with CB_1-expressing CHO cells or CHO cell membranes, are attenuation of [^{35}S]GTPγS binding (Table 7) [59, 60, 66], inhibition of basal MAP kinase activity [59] and enhancement of forskolin-stimulated cyclic AMP production [42, 59]. Such effects of SR141716A have not been detected in experiments with untransfected CHO cell preparations [59, 66]. In some experiments [62, 67], though not in others [64], SR141716A has also been reported to produce a slight attenuation of [^{35}S]GTPγS binding to rat cerebellar membranes, albeit at 10 μM and not at lower concentrations. Other inverse cannabimimetic effects of SR141716A observed in CB_1 receptor-containing preparations, are enhancement of (a) evoked acetylcholine release in rat hippocampal slices [155, 156], (b) evoked noradrenaline release in guinea-pig hippocampal slices and retinal discs [157, 158], (c) evoked acetylcholine release in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine [159] and (d) evoked contractions of guinea-pig intestinal and mouse urinary bladder preparations [131, 160, 161]. At the rather high concentration of 1 μM, SR141716A has also been reported to decrease endothelium-dependent carbachol- and A23187-induced reversal of vasomotor tone produced by methoxamine in rat endothelium-intact small mesenteric arteries [162], vasorelaxation induced in this preparation by levcromakalim [163], endothelium-dependent vasorelaxation induced by carbachol or A23187 in rat isolated perfused superior mesenteric arterial bed [164, 165] and endothelium-dependent reductions in coronary perfusion pressure induced by bradykinin in rat isolated perfused heart [166].

The discovery that SR141716A can produce inverse cannabimimetic effects in some biological systems suggests that the endogenous cannabinoid system is tonically active in these systems. This could be because these biological systems are releasing an endogenous CB_1 receptor agonist that produces cannabimimetic tone susceptible to attenuation by SR141716A. Another possibility is that cannabinoid CB_1 receptors can exist in two interchangeable states, the one precoupled to and the other uncoupled from the effector system. It could then be that SR141716A shows activity by itself because it is an inverse agonist rather than a pure antagonist, binding preferentially to the receptors in the uncoupled state and so shifting the equilibrium away from the receptors in the precoupled state. These hypotheses are not mutually exclusive. Indeed, it is possible that tonic activity of the endogenous cannabinoid system stems mainly from endogenous cannabinoid release in some biological systems or experimental protocols, from receptor precoupling in others and from a combination of both mechanisms in yet a third group of biological systems or protocols.

Bouaboula *et al.* [59] concluded that in their experiments with CB_1 transfected CHO cells, SR141716A did not induce inverse cannabimimetic effects by blocking the actions of endogenous cannabinoids. This conclusion was based in part on their observation that whilst GTPγS decreased the affinity of CP55940 for CB_1 receptors it increased SR141716A affinity for these receptors, the expectation being that guanidylic nucleotide analogues such as GTPγS will decrease agonist binding and increase inverse agonist binding. They also found that the fluid bathing the transfected CHO cells did not contain material with detectable cannabimimetic activity. In addition, they showed SR141716A to be markedly less potent in reversing the inhibitory effect of CP55940 on forskolin stimulated cyclic AMP production than in inducing inverse cannabimimetic effects (including enhancement of forskolin stimulated cyclic AMP production) when administered alone. They argued that this large potency difference would not have existed if SR141716A had produced its inverse cannabimimetic effects by blocking the actions of endogenous cannabinoids. There was also good agreement between the concentrations at which SR141716A induces inverse cannabimimetic effects and those at which it binds to CB_1 receptors.

MacLennan *et al.* [66] also concluded that SR141716A acted as an inverse agonist in their experiments with CB_1 transfected CHO cells. They found that the inverse cannabimimetic effect of SR141716A on [^{35}S]GTPγS binding to membranes of these cells was not mimicked by phenylmethylsulphonyl fluoride (section above), applied at a concentration expected inhibit the enzymic hydrolysis of anandamide (100 μM). Nor was [^{35}S]GTPγS binding inhibited (or stimulated) by cannabinol at a concentration (10 μM) 100 fold higher than its K_i value for displacement of [^{3}H]CP55940 from CB_1 binding sites. It is worth noting that even though cannabinol had no effect at all on [^{35}S]GTPγS binding in these experiments, other studies have shown it to behave as a partial agonist at CB_1 receptors rather than as a pure antagonist in both the [^{35}S]GTPγS assay and the cyclic AMP assay (see above and Tables 3 and 4).

Data obtained by Pertwee *et al.* [167] in experiments with the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine also support the hypothesis that SR141716A is an inverse agonist. They found that although phenylmethylsulphonyl fluoride potentiates anandamide-induced inhibition of electrically-evoked

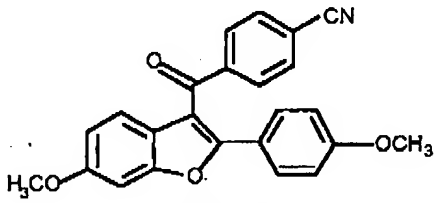
contractions of this preparation, it does not share the ability of SR141716A to enhance such contractions when administered by itself. Not all CB_1-containing systems show signs of tonic activity when exposed to SR141716A (e.g. [64, 101, 158, 168-170]). Whether this reflects an absence from these biological systems of sufficient precoupled receptors and/or of sufficient ongoing endogenous cannabinoid production remains to be established.

SR144528

SR144528 (Fig. 5) was recently developed by Sanofi Recherche as a selective CB_2 receptor antagonist [43]. It binds with markedly higher affinity to CB_2 than CB_1 receptors (Table 2), shows no significant affinity for a wide range of non-cannabinoid receptors [43] and prevents the production of CB_2 receptor-mediated in vitro effects with a potency not far below that at which it displaces [^{3}H]CP55940 from CB_2 binding sites. Thus, in human CB_2 transfected CHO cells, SR144528 readily reverses both CP55940-induced inhibition of forskolin-stimulated cyclic AMP production (EC_{50} = 10 nM) and CP55940-induced stimulation of MAP kinase (EC_{50} = 39 nM) [43]. In addition, orally administered SR144528 has been shown to produce a dose-related inhibition of in vitro specific binding of [^{3}H]CP55940 to membranes of mouse spleen, a tissue that contains a much higher population of CB_2 than CB_1 receptors [43]. None of the doses of SR144528 administered in this investigation affected [^{3}H]CP55940 binding to CB_1 receptors on mouse brain membranes. Although SR144528 is CB_1-selective it is not CB_1-specific (Table 2) and there is evidence that at micromolar and high nanomolar concentrations it can block CB_1 receptor-mediated effects [43]. When administered by itself, SR144528 resembles SR141716A in being able to produce effects that are opposite in direction from those produced by cannabinoid receptor agonists. Thus there are reports that in CHO cells or CHO cell membranes containing human CB_2 receptors, SR144528 potently inhibits [^{35}S]GTPγS binding (Table 7) and enhances forskolin-stimulated cyclic AMP production [43, 47]. SR141716A can also act through CB_2 receptors to inhibit [^{35}S]GTPγS binding [66] when administered at the rather high concentrations at which it is expected to undergo significant binding to this receptor type. One inverse cannabimimetic effect that SR144528 has been shown to produce in vivo is hyperalgesia [124].

LY320135

This ligand (Fig. 14), developed by Eli Lilly as a selective CB_1 receptor antagonist, has been reported to oppose the production of several CB_1 receptor-mediated in vitro effects: (+)-WIN55212-induced inhibition of N-type calcium current and activation of inwardly rectifying potassium current and anandamide and (+)-WIN55212-induced inhibition of forskolin-stimulated cyclic AMP production [42]. It is also active in vivo, a dose of 20 mg/kg i.p. producing antagonism of the sedation produced by anandamide in mice subjected to an open-field test [50]. LY320135 has less affinity for CB_1 receptors than SR141716A but, like SR141716A, binds with significantly higher affinity to CB_1 than CB_2 receptors (Table 2). At concentrations below 10 μM, LY320135 also displaces ligands from muscarinic receptors (K_i = 2.1 μM) and $5HT_2$ receptors (K_i = 6.4 μM) although not from α-adrenoceptors, dopamine D_1 or D_2 receptors, benzodiazepine receptors, histamine H_1 receptors or γ-aminobutyric acid receptors (K_i > 10 μM) [42]. When administered by itself, LY320135 has been found to have little effect on calcium or potassium currents. However, it has been reported to enhance forskolin-induced stimulation of cyclic AMP production by CHO cells expressing CB_1 receptors [42]. The basis of this inverse cannabimimetic effect remains to be established.



LY320135

Fig. (14). Structure of LY320135.

General Discussion and Summary

It is clear from the material presented in this review that potent antagonists/inverse agonists with marked selectivity for CB_1 or CB_2 receptors are now available. These constitute powerful experimental tools for exploring the physiology and pharmacology of the endogenous cannabinoid system. Other important recent advances are the development of agonists with improved selectivity for CB_1 or CB_2 receptors and the emergence of clues as to how the structural features of CB_1 and CB_2 receptor ligands that govern affinity differ from those that determine efficacy at these receptors. Far less is known about the structure-activity relationships of efficacy than of affinity. However, the recent development of the [^{35}S]GTPγS assay and availability of an irreversible antagonist (methyl arachidonyl fluorophosphonate) should facilitate efficacy studies in the future. CB_1 and CB_2 receptor agonists can vary widely in their efficacies. It is particularly noteworthy that Δ^9-THC behaves as a partial agonist at CB_1 receptors and that it seems to have even less efficacy at CB_2 receptors as this implies that data obtained from experiments with Δ^9-THC (or cannabis)

will underestimate the consequences of maximal CB_1 and CB_2 receptor stimulation in the whole organism. It will be important to establish whether the partial agonism that Δ^9-THC shows at CB_1 receptors confers any clinical benefits by avoiding or reducing the intensity of certain adverse effects so as to improve the benefit to risk ratio. Although anandamide has greater efficacy than Δ^9-THC at CB_1 receptors, it too behaves as a very low efficacy agonist at the CB_2 receptor, raising the possibility that some other endogenous compound serves as the natural agonist for this receptor type.

From the available evidence it seems likely that CB_1 and CB_2 receptors can exist in a precoupled state, at least in the high-expression systems of transfected cell, and that agents such as SR141716A, SR144528 LY320135 and AM630 behave as inverse agonists rather than as pure antagonists in such systems. Whether they also act in this way in tissues endowed with their own CB_1 or CB_2 receptor expression systems will depend on the extent to which cannabinoid receptor precoupling occurs in these 'more physiological' systems, the possibility remaining that the production of inverse cannabimimetic effects in such tissues may also stem from an ability to block the actions of released endogenous cannabinoids. Whatever the underlying mechanisms, the observation that inverse cannabimimetic effects can be produced by cannabinoid receptor ligands in some tissues that naturally express CB_1 or CB_2 receptors constitutes evidence that the endogenous cannabinoid system is tonically activity in these tissues. Further experiments are now required to elucidate the basis of this tonic activity, which has been detected both in vitro and in vivo, and to identify any disease states in which this activity changes. It will also be important to establish whether it is possible to develop pure antagonists that lack the ability to produce inverse cannabimimetic effects in tissues in which there is no ongoing release of endogenous cannabinoids.

As to possible clinical uses for cannabinoid receptor agonists, these are detailed elsewhere [7, 171]. They include the management of glaucoma, bronchial asthma and pain and the suppression of muscle spasticity/spasm associated with conditions such as multiple sclerosis and spinal cord injury. CB_1 receptor antagonists/inverse agonists also have therapeutic potential as appetite suppressants [50, 149, 150, 152] as well as in the management acute schizophrenia and/or for ameliorating cognitive/memory dysfunctions associated with disorders such as Alzheimer's disease [50]. An answer to the question of whether drugs that selectively activate or block CB_2 receptors have therapeutic potential, for example as immunomodulators, must await a more complete characterization of this component of the endogenous cannabinoid system.

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